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(54) Title: IDENTIFICATION AND EXPRESSION OF INSECT STEROID RECEPTOR DNA SEQUENCES

(57) Abstract

The present invention involves the isolation of insect DNA sequences having characteristics of insect steroid receptors. Also described is the putative amino acid sequence for insect steroid receptors as deduced from the DNA sequence.

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features. Anatomic similarity is a reflection of developmental, physiological and biochemical similarities shared by these insects. In particular, the principles governing the role of insect ecdysteroid-hormone receptors in _Drosophila_ development, as described above, likely are shared by many different types of insects.

As one weapon against the destruction of cultivated crops by insects, organic molecules with pesticidal properties are used commonly in attempts to eliminate insect populations. However, the ecological side effects of these pesticides, due in part to their broad activity and lack of specificity, and also in part to the fact that some of these pesticides are not easily biodegradable, significantly affect populations of both insects and other species of animals. Some of these populations may be advantageous from an ecological or other perspective. Furthermore, as the insect populations evolve to minimize the effects of the applied pesticides, greater amounts of pesticides must be applied, causing significant direct and indirect effects on other animals, including humans. Thus, an important need exists for both highly specific and highly active pesticides which are biodegradable. Novel insect hormones which, like the ecdysteroids, act by complexing with insect members of the steroid receptor superfamily to control insect development, are likely candidates for pesticides with these desirable properties.

The use of insect hormones may also have other important applications. Many medically and commercially important proteins can be produced in a usable form by genetically engineered bacteria. However, many expressed proteins are processed incorrectly in bacteria and are preferably produced by genetically engineered eucaryotic cells. Typically, yeast cells or mammalian tissue-culture cells are used. Because it has been observed that protein processing of foreign proteins in yeast cells is also frequently inappropriate, mammalian

cultured cells have become the central focus for the production of many proteins. It is commonly known that the production of large amounts of foreign proteins makes these cells unhealthy, which may affect adversely the yield of the desired protein. This problem may be circumvented, in part, by using an inducible expression system. In such a system, the cells are engineered so that they do not express the foreign protein until an inducing agent is added to the growth medium. In this way, large quantities of healthy cells can be produced and then induced to produce large amounts of the foreign protein. Unfortunately, in the presently available systems, the inducing agents themselves, such as metal ions or high temperature, adversely affect the cells, thus again lowering the yield of the desired foreign protein the cells produce. A need therefore exists for the development of benign inducing factors for efficient production of recombinant proteins. Such factors could also prove invaluable for the therapy of human patients suffering from inability to produce particular proteins, treatment with these factors controlling both the timing and the abundance of the protein produced in the affected individual.

The hormones that complex with mammalian or other vertebrate members of the steroid receptor superfamily are unlikely candidates as such benign factors because they would alter the expression of many target genes in cells bearing these receptors, thereby adversely affecting the host cells.

For these and other reasons, obtaining steroid receptors or nucleic acid information about them has been a goal of researchers for several years. Unfortunately, efforts have been unsuccessful despite a significant investment of resources. The absence of information on the structure and molecular biology of steroid receptors has significantly hindered the ability to produce such products.

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Thus, there exists a need for detailed sequence information on insect members of the steroid receptor superfamily, and the genes that encode these receptors and for resulting reagents. Reagents are provided which are useful in finding new molecules which may act as agonists or antagonists of natural insect members of the steroid receptor superfamily, or as components of systems for highly specific regulation of recombinant proteins in mammalian cells.

SUMMARY OF THE INVENTION

In accordance with the present invention, isolated recombinant nucleic acids are provided which, upon expression, are capable of coding for other than a native vertebrate steroid receptor or fragment thereof. These nucleic acids typically comprise a segment having a sequence substantially homologous to one or more coding regions of domains A, B, D, E, or F from an insect steroid receptor superfamily member gene having steroid binding domain homology, e.g., EcR, DHR3, E75A, or E75B. Preferably, the nucleic acids encode a polypeptide capable of binding to a ligand for an insect steroid receptor superfamily member and are capable of hybridizing to an insect steroid receptor superfamily member gene segment under selective hybridization conditions, usually stringent hybridization conditions. Mammalian cells transformed with the nucleic acids are also provided.

In another embodiment, isolated recombinant nucleic acids are included that have sequence exhibiting identity over about 20 nucleotides of a coding segment of an insect steroid receptor superfamily member having steroid binding domain homology. The nucleic acids can be transformed into cells to express a polypeptide which binds to a control element responsive to a ligand of an insect steroid receptor superfamily.

Alternatively, an isolated DNA molecule is provided comprising a DNA sequence capable of binding to an insect steroid receptor superfamily member other than 20-OH ecdysone receptor, such as DHR3, E75A, or E75B. The DNA sequence will generally be present in an expression vector and promote transcription of an operably linked sequence (e.g., encoding a polypeptide) in response to binding by an insect steroid receptor superfamily member. Cells comprising the nucleic acids are provided, as are cells expressing the polypeptides. In certain embodiments, non-insect cells will be used, including mammalian cells. Also contemplated are recombinant nucleic acids comprising a controlling element responsive to a ligand, e.g., a ligand which binds to an insect steroid receptor superfamily member ligand responsive controlling element, a non-heat shock promoter sequence (e.g., an alcohol dehydrogenase promoter) and a sequence comprising a reporter gene. Usually the controlling element will operate to make transcription of the reporter gene responsive to the presence of the ligand.

Additional embodiments of the present invention include polypeptides comprising an insect steroid receptor superfamily member or fragment thereof, wherein such polypeptide is substantially free of naturally-associated insect cell components and exhibits a biological activity characteristic of an insect steroid receptor superfamily member with a hormone binding domain. Preferably, the insect steroid receptor superfamily member or fragment thereof also comprises a DNA binding domain and the polypeptide is capable of binding to a hormone analogue selected from the group consisting of an insect hormone, an insect hormone agonist and an insect hormone antagonist. The polypeptide can comprise a zinc-finger domain and usually is capable of binding to a DNA controlling element responsive to an insect hormone. As desired, the polypeptide will be fused to a second polypeptide,

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typically a heterologous polypeptide which comprises a
second steroid receptor superfamily member. Cells, often
mammalian cells, comprising the protein are provided.

5   Fragments of such polypeptides can have a sequence
substantially homologous to consensus E1, E2 or E3 region
sequences. By way of example, a preferred fragment has a
sequence comprising:

a segment at least about 25% homologous to a
consensus E1 region sequence;

10   a segment at least about 30% homologous to a
consensus E2 region sequence; and
a segment at least about 30% homologous to a
consensus E3 region sequence.

The polypeptides of the present invention have a

15   variety of utilities. For example, a method for
selecting DNA sequences capable of being specifically
bound by an insect steroid receptor superfamily member
can comprise the steps of screening DNA sequences for
binding to such polypeptides and selecting DNA sequences

20   exhibiting such binding. Alternatively, methods for
selecting ligands, e.g., ecdysteroid analogues, specific
for binding to a hormone binding domain of an insect
steroid receptor superfamily member can comprise the
steps of screening compounds for binding to one or more

25   superfamily members and selecting compounds exhibiting
specific binding to the members.
Also included are methods for modulating insect
physiology or development (e.g., killing) comprising the
steps of screening compounds for binding to an insect

30   steroid receptor superfamily member, selecting compounds
exhibiting said binding and administering the ligand to
an insect.

Additionally provided are methods for selecting
ligands specific for binding to a ligand binding domain

35   of an insect steroid receptor superfamily member
comprising combining:

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> (i)   a fusion polypeptide which comprises a
>   ligand binding domain functionally
>   linked to a DNA binding domain of a
>   second steroid receptor superfamily
>   member; and
> (ii) a second nucleic acid sequence encoding a
>   second polypeptide, wherein
>   expression of the second nucleic acid
>   sequence is responsive to binding by
>   the DNA binding domain;

screening compounds for an activity of inducing
expression of the second polypeptide; and
selecting those compounds which do so.

This will often be performed in a cell, e.g., with cells
transformed with DNA encoding a fusion protein. This
method allows selction of analogues which are useful in
modulating insect physiology or development.

Also provided are methods for producing a
polypeptide comprising the steps of:

> selecting a cell, typically a mammalian or plant
>   cell which is substantially insensitive to
>   exposure of an insect steroid receptor
>   superfamily ligand;
> introducing into said cell:
> (i)   a receptor for the ligand; and
> (ii) a nucleic acid sequence encoding the
>   polypeptide, the nucleic acid
>   sequence operably linked to a
>   controlling element responsive
>   to presence of the selected
>   ligand, wherein a transformed
>   cell is produced; and

exposing the transformed cell to the ligand.

Usually the cell will be a mammalian cell, and will
sometimes be introduced into a whole organism, e.g., a
plant or animal.

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Kits for the determination of expression levels of the nucleic acids and proteins provided herein are made available. Typically, the kit will have at least one compartment which contains a reagent which specifically binds to the desired target molecule, e.g., ligand analogues, receptors, or nucleic acids.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. pMTEcR, a Cu^{2+}-inducible EcR expression plasmid. The P_{MT}, EcR ORF and Act 5c poly A elements are defined in Experimental Example III, part A. The HYG^r ORF confers hygromycin resistance and is under control of the promoter in the LTR of Drosophila transposable elements, copia. The SV40 intron/poly A element provides an intron for a possible splicing requirement, as well as a polyadenylation/cleavage sequence for the HYG^r ORF mRNA. The pAT153 DNA derives from a bacterial plasmid.

Figure 2. The ecdysone-inducible pEcRE/Adh/βgal reporter plasmid. See the text of Experimental Example III, part B, for the construction of this plasmid and the definitions of all symbols (except the SV40 splice and poly A) which are defined in the figure legend.

Figure 3. The constitutive EcR expression plasmid, pActEcR. The construction of this plasmid and the definition of the symbols are given in Experimental Example III, part B.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides novel isolated nucleic acid sequences encoding polypeptide products exhibiting the structure and/or activities of insect members of the steroid receptor superfamily. Having elucidated the structures of these insect steroid receptors from their genes, the separate ligand-binding domains and DNA-binding domains are used individually or in combination to screen for new ligands or DNA sequences which bind to these domains. Thus, for example, by

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binding to promotor sequences incorporating a DNA binding
site, these receptors will usually control expression of
reporter genes for which sensitive assays exist. Or, the
hormone-binding domains serve as reagents for screening
for agonists or antagonists of steroid receptor
superfamily members. Either new classes of molecules, or
selected modifications of known ligands will be screened
for receptor binding. New ligands obtained in this way
find use as highly specific and highly active naturally
occurring pesticides. Alternatively, structural
information about interactions between the ligand and
binding domains directs methods for mutagenizing or
substituting particular residues in the binding domains,
thereby providing for altered binding specificity. Thus,
inter alia, the present invention provides for screening
for new ligand molecules, for the design of new ligand-
binding domain interactions, for producing novel chimeric
steroid receptor superfamily members and for generating
new combinations of ligands and binding domains.

The present invention also provides for the
isolation or identification of new steroid hormone-
responsive elements and associated genes. By appropriate
operable linkage of selected sequences to DNA controlling
elements which are responsive to binding by the DNA-
binding domains of steroid receptor superfamily members,
new regulatory combinations result. The present
invention further provides for the design of either a
binding domain in a member of the insect steroid receptor
superfamily that will recognize given DNA sequences, or
conversely for the modification of DNA sequences which
will bind to particular receptor DNA-binding domains.
Both the DNA-binding domain of a superfamily-member
polypeptide and its DNA recognition sequence can be
coordinately modified to produce wholly new receptor-DNA
interactions.

In an alternative embodiment, a DNA-binding sequence
recognized by a selected receptor will be operably linked

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to a desired genetic sequence for inducible expression.
Thus, upon administration of a ligand specific for that
selected receptor, the genetic sequence is appropriately
regulated. Expression systems are constructed that are
responsive to administration of insect steroid receptor
superfamily-specific ligands. By identifying and
isolating new members of the insect steroid receptor
superfamily, new, useful regulatory reagents become
available, both hormones and controlling elements.

In another embodiment, highly regulatable expression
of a gene is achieved by use of regulatory elements
responsive to ligands specific to the superfamily
members. If transformed cells are grown under conditions
where expression is repressed or not induced, the cells
will grow to higher densities and enjoy less stressful
conditions. Upon reaching high density, the regulatory
ligand molecule is added to cause high expression.
Selected cells otherwise insensitive to the inducing
ligand will not be affected by exposure to the ligand
used to regulate expression. This provides a means both
for highly efficient regulatable expression of genes, and
for introduction of these genes into intact organisms.

In accordance with specific embodiments of the
present invention, nucleic acid sequences encoding
portions of insect steroid hormone receptor superfamily
members have been elucidated. DNA encoding four
different members of the Drosophila steroid receptor
superfamily have been characterized: (1) the 20-OH
ecdysone receptor, also called the ecdysone receptor
(EcR), for which a full-length encoding sequence has been
determined; (2) Drosophila hormone receptor 3 (DHR3), a
protein with sequence homology to various steroid
receptor superfamily members; (3 and 4) E75A and E75B,
closely related proteins, encoded by segments of the same
gene, and each possessing sequence homology to other
steroid receptor superfamily members.

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The DNA sequences encoding each of these members of the insect steroid receptor superfamily provide probes for screening for homologous nucleic acid sequences, both in _Drosophila_ and other sources. This screening allows

5   isolation of homologous genes from both vertebrates and invertebrates. Production of large amounts of the encoded proteins is effected by inserting those sequences into expression systems.

The EcR, DHR3, E75A, and E75B genes are each linked

10   to similar DNA sequences which likely function as controlling, or regulatory elements which are responsive to insect steroids. The present invention provides for the isolation of these hormone-responsive control elements, and for their use in regulating gene

15   expression. One embodiment of a DNA construct comprises: (1) multiple copies of an insect steroid receptor superfamily controlling element linked to (2) a minimal gene promoter, preferably not a heat shock gene promoter, which provides highly inducible expression of (3) an

20   operably linked gene. This construct provides a very sensitive assay for the presence of the controlling molecule of the receptor.

Another aspect of the present invention involves cells comprising: (1) isolated recombinant gene segments

25   encoding biologically active fragments of insect steroid receptor superfamily proteins; (2) DNA sequences which bind insect steroid receptors, e.g., the elements involved in hormone-responsive control; or (3) modified receptor proteins. Transformed cells are understood to

30   include their progeny. In particular, the present invention provides for a system whereby expression of polypeptides is responsive to steroid induction. For instance, a system which expresses a desired protein in response to exposure to ecdysone analogues is constructed

35   by operably linking a promoter having an ecdysone-responsive enhancer to a peptide encoding segment.

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The present invention also provides insect steroid receptor proteins substantially free from naturally-associated insect cell components. Such receptors will typically be either full-length proteins, functional fragments, or fusion proteins comprising segments from an insect steroid receptor protein fused to a heterologous, or normally non-contiguous, protein domain.

The present invention further provides a number of methods for utilizing the subject receptor proteins. One aspect of the present invention is a method for selecting new hormone analogues. The isolated hormone-binding domains specifically bind hormone ligands, thereby providing a means to screen for new molecules possessing the property of binding with high affinity to the ligand-binding region. Thus, a binding domain of an insect steroid receptor superfamily member will be used as a reagent to develop a binding assay. On one level, the binding domains are useful as affinity reagents for a batch or a column selective process, i.e., to selectively retain ligands which bind. Alternatively, a functional assay is preferred for its greater sensitivity to ligand-binding, whether a direct binding assay or an indirect assay in which binding is linked to an easily assayed function. For example, by operable linkage of an easily assayable reporter gene to a controlling element responsive to binding by an insect steroid receptor superfamily member, in which ligand-binding induces protein synthesis, an extremely sensitive assay for the presence of a ligand or of a receptor results. Such a construct useful for assaying the presence of 20-OH ecdysone is described below. This construct is useful for screening for agonists or antagonists of the 20-OH ecdysone ligand.

In particular, this method allows selecting for ligands which bind to an "orphan" receptor, i.e., a receptor whose ligand is unknown. Binding domains for "unknown" ligands will often originate from either newly

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identified insect steroid receptor superfamily members, or from mutagenesis. A hybrid receptor will be created with a ligand-binding domain and DNA-binding domain from different sources. For example, a hybrid receptor between a putative binding domain and a known DNA-binding domain would allow screening for ligands. An "orphan receptor" binding domain will be functionally linked to a known DNA-binding domain which will control a known reporter gene construct whose expression will be easily detected. This system for ligand-receptor binding provides an extremely sensitive assay for ligand-receptor interactions.

Alternatively, the recognition of important features of tertiary structure and spatial interactions between a ligand-binding domain from an insect steroid receptor superfamily member and its ligand will allow selection of new combinations of ligand-binding domains with ligands. Either method provides for selecting unusual ligands which specifically bind a modified polypeptide-binding domain of a receptor. This approach allows selection of novel steroid hormone analogues which exhibit modified specificity for binding to a subgroup of steroid receptors.

The present invention also provides for new and useful combinations of the various related components: the recombinant nucleic acid sequences encoding the polypeptides, the polypeptide sequences, and the DNA sites to which the receptors bind (i.e., the regulatory, or control, elements). For instance, fusing portions of nucleic acid sequences encoding peptides from different sources will provide polypeptides exhibiting hybrid properties, e.g., unusual control and expression characteristics. In particular, hybrid receptors comprising segments from other members of the superfamily, or from other sources, will be made. Combining an insect steroid receptor-responsive enhancer segment with a different polypeptide encoding segment

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will produce a steroid-responsive expression system for that polypeptide.

The isolation of insect steroid receptors provides for isolation or screening of new ligands for receptor binding. Some of these will interfere with, or disrupt, normal insect development. These reagents will allow the user to accelerate or decelerate insect development, for instance, in preparing sterile adults for release. Alternatively, a delay or change in the timing of development will often be lethal or will dramatically modify the ability of an insect to affect an agricultural crop. Thus, naturally occurring biodegradable and highly active molecules able to disrupt the timing of insect development will result.

Furthermore, these polypeptides provide the means by which have been raised antibodies possessing specificity for binding to particular steroid receptor classes. Thus, reagents will be produced for determining, qualitatively or quantitatively the presence of these or homologous polypeptides. Alternatively, these antibodies will be used to separate or purify receptor polypeptides.

Transcription sequences of insect steroid receptor superfamily members

The ecdysone receptor gene is a member of the steroid and thyroid hormone receptor gene superfamily, a group of ligand-responsive transcription factors. See, Evans (1988) Science 240:889-895; and Segraves, Molecular and Genetic Analysis of the E75 Ecdysone-Responsive Gene of Drosophila melanogaster (Ph.D. thesis, Stanford University 1988), both of which are hereby incorporated herein by reference for all purposes. These receptors show extensive sequence similarity, especially in their "zinc finger" DNA-binding domains, and also in a ligand (or hormone or steroid) binding domain. Modulation of gene expression apparently occurs in response to binding of a receptor to specific control, or regulatory, DNA

elements. The cloning of receptor cDNAs provides the
first opportunity to study the molecular bases of steroid
action. The steroid receptor superfamily is a class of
receptors which exhibit similar structural and functional
features. While the term insect is used herein, it will
be recognized that the same methods and molecules will be
derived from other species of animals, in particular,
those of the class Insecta, or, more broadly, members of
the phylum Arthropoda which use ecdysteroids as hormones.
Members of the insect steroid receptor superfamily are
characterized by functional ligand-binding and DNA
binding domains, both of which interact to effect a
change in the regulatory state of a gene operably linked
to the DNA-binding site of the receptor. Thus, the
receptors of the insect steroid receptor superfamily seem
to be ligand-responsive transcription factors. The
receptors of the present invention exhibit at least a
hormone-binding domain characterized by sequence homology
to particular regions, designated E1, E2 and E3.

The members of the insect steroid receptor
superfamily are typically characterized by structural
homology of particular domains, as defined initially in
the estrogen receptor. Specifically, a DNA-binding
domain, C, and a ligand-binding domain, E, are separated
and flanked by additional domains as identified by Krust
et al. (1986) EMBO J. 5:891-897, which is hereby
incorporated herein by reference.

The C domain, or zinc-finger DNA-binding domain, is
usually hydrophilic, having high cysteine, lysine and
arginine content -- a sequence suitable for the required
tight DNA binding. The E domain is usually hydrophobic
and further characterized as regions E1, E2 and E3. The
ligand-binding domains of the present invention are
typically characterized by having significant homology in
sequence and structure to these three regions. Amino
proximal to the C domain is a region initially defined as
separate A and B domains. Region D separates the more

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conserved domains C and E. Region D typically has a
hydrophilic region whose predicted secondary structure is
rich in turns and coils. The F region is carboxy
proximal to the E region (see, Krust et al., supra).

5   The ligand-binding domain of the members of the
insect steroid receptor superfamily is typically
carboxyl-proximal, relative to a DNA-binding domain
described below. See, Evans (1988) Science 240:889-895.
The entire hormone-binding domain is typically between
10   about 200 and 250 amino acids but is potentially shorter.
This domain has the subregions of high homology,
designated the E1, E2 and E3 regions. See, e.g.,
Table 4.

The E1 region is 19 amino acids long with a
15   consensus sequence AKX(L/I)PGFXXLT(L/I)(D/E)DQITLL, where
X represents any amino acid and the other letters are the
standard single-letter code. Positions in parentheses
are alternatives. Typically, members of the insect
steroid receptor superfamily will have at least about
20   five matches out of the sixteen assigned positions,
preferably at least about nine matches, and in more
preferred embodiments, at least about ten matches.
Alternatively, these insect steroid receptor superfamily
members will have homologous sequences exhibiting at
25   least about 25% homology, normally at least about 30%
homology, more normally at least about 35% homology,
generally at least about 40% homology, more generally at
least about 45% homology, typically at least about 50%
homology, more typically at least about 55% homology,
30   usually at least about 60% homology, more usually at
least about 70% homology, preferably at least about 80%
homology, and more preferably at least about 90% homology
at positions assigned preferred amino acids.

The E2 region is a 19 amino-acid segment with a
35   consensus sequence:

E(F/Y)(A/V)(L/C)(L/M)KA(I/L)(V/L)L(L/I)

(N/S)(S/P)D(P/-)(R/K)(P/D)GL   ,

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where - represents an optional absence of an amino acid.
Typically, an insect steroid receptor superfamily member
will exhibit at least about six matches, preferably at
least about eight matches and more preferably at least
about nine matches. Alternatively, E2 sequences of
insect steroid receptor superfamily members exhibit at
least about about 25% homology, normally at least about
30% homology, more normally at least about 35% homology,
generally at least about 40% homology, more generally at
least about 45% homology, typically at least about 50%
homology, more typically at least about 55% homology,
usually at least about 60% homology, more usually at
least about 70% homology, preferably at least about 80%
homology, and more preferably at least about 90% homology
at positions assigned preferred amino acids.

The E3 region is a 12 amino-acid segment with a
consensus sequence

LXKLLXXLPDLR .

The insect steroid receptor superfamily members will
typically show at least about four matches out of the
nine assigned preferences in the E3 region, preferably at
least about five matches and more preferably at least
about six matches. Alternatively, over the assigned
positions, members of the insect steroid receptor
superfamily will exhibit at least about 25% homology,
normally at least about 30% homology, more normally at
least about 35% homology, generally at least about 40%
homology, more generally at least about 45% homology,
typically at least about 50% homology, more typically at
least about 55% homology, usually at least about 60%
homology, more usually at least about 70% homology,
preferably at least about 80% homology, and more
preferably at least about 90% homology at positions
assigned preferred amino acids.

In preferred embodiments, the insect steroid
receptor superfamily members will exhibit matching of at
least about five positions in an E1 region, at least

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about six positions in an E3 region and at least about four positions in an E3 region. The E1, E2, and E3 regions are defined, e.g., in Table 4.

The DNA-binding domain of these insect steroid receptor superfamily members is characterized by a "zinc fingers" motif. See, Evans (1988) Science 240:889-895. The domain is typically amino proximal to the ligand, or hormone, binding site. Typically, the DNA-binding domain of the insect steroid receptor superfamily members is characterized by clustering of basic residues, a cysteine-rich composition and sequence homology. See, Evans, R. M. (1988) Science 240:889-89; and Experimental section below. Significant polypeptide sequence homology among superfamily members exists. The insect steroid receptor superfamily members will exhibit at least about 30% homology in the 67 $\pm$ 1 amino acid region of this domain, normally at least about 40% homology, usually at least about 45% homology, and preferably at least about 55% homology.

Steroids are derivatives of the saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene. Among the molecules in the group "steroids" are: the bile acids, e.g., cholic acid, and deoxycholic acid; the adrenocortical steroids, e.g., corticosterone and aldosterone; the estrogens, e.g., estrone and β-estradiol; the androgens, e.g., testosterone and progesterone; and the ecdysteroids. The terms steroid or steroid hormones are used interchangeably herein and are intended to include all steroid analogues. Typically, steroid analogues are molecules which have minor modifications of various peripheral chemical groups. See, Koolman (ed.) (1989), cited above, for details on ecdysteroids.

Although ligands for the insect steroid receptor superfamily members have historically been characterized as steroids, the term "steroid" as in "insect steroid receptor superfamily" is not meant only literally. The

use of "steroid" has resulted from a historical
designation of members of a group recognized initially to
include only molecules having specific defined molecular
structures. However, this limitation is no longer
applicable since functions are no longer only correlated
with precise structures. Thus, there will be members of
the insect steroid receptor superfamily, as defined
herein, whose ligand-binding specificities are not
directed to classically defined "steroids." Typically,
the ligands for members of the insect steroid receptor
superfamily are lipophilic molecules which are structural
analogues of steroid molecules.

The term ligand is meant to refer to the molecules
that bind the domain described here as the "hormone-
binding domain." Also, a ligand for an insect steroid
receptor superfamily member is a ligand which serves
either as the natural ligand to which the member binds,
or a functional analogue which serves as an agonist or
antagonist. The classical definition of "hormone" has
been defined functionally by physiologists, see, e.g.,
Guyton, Textbook of Medical Physiology, Saunders,
Philadelphia. The functional term "hormone" is employed
because of historic usage, but is meant to apply to other
chemical messengers used to communicate between cell
types. Recently the distinction between hormones and
neurotransmitters has been eroded as various peptide
neurotransmitters have been shown to exhibit properties
of classically defined hormones. These molecules are
typically used in intercellular signal transduction, but
are not limited to those molecules having slow or
systemic effects, or which act at remote sites.

Substantial homology in the nucleic acid context
means either that the segments, or their complementary
strands, when compared, are identical when optimally
aligned, with appropriate nucleotide insertions or
deletions, in at least about 40% of the residues,
generally at least about 45%, more generally at least

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about 50%, normally at least about 55%, more normally at least about 60%, typically at least about 65%, more typically at least about 70%, usually at least about 75%, more usually at least about 80%, preferably at least about 85%, and more preferably at least about 95% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand or its complement, typically using a sequence derived from Table 1, 2 or 3. Selectivity of hybridization exists when hybridization occurs which is more selective than total lack of specificity. Normally, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 to 25 nucleotides, generally at least about 65%, typically at least about 75%, usually at least about 85%, preferably at least about 90%, and more preferably at least about 95% or more. See, Kanehisa, M. (1984), Nucleic Acids Res. 12:203-213, which is incorporated herein by reference. Stringent hybridization conditions will include salt concentrations of less than about 2.5 M, generally less than about 1.5 M, typically less than about 1 M, usually less than about 500 mM, and preferably less than about 200 mM. Temperature conditions will normally be greater than 20°C, more normally greater than about 25°C, generally greater than about 30°C, more generally greater than about 35°C, typically greater than about 40°C, more typically greater than about 45°C, usually greater than about 50°C, more usually greater than about 55°C, and in particular embodiments will be greater than 60°C, even as high as 80°C or more. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure of any one.

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A gene for an insect steroid receptor superfamily member gene includes its upstream (e.g., promoter) and downstream operably linked controlling elements, as well as the complementary strands. See, generally, Watson et al. (1987) The Molecular Biology of the Gene, Benjamin, Menlo Park, which is hereby incorporated herein by reference. A gene geneally also comprises the segment encoding the transcription unit, including both introns and exons. Thus, an isolated gene allows for screening for new steroid receptor genes by probing for genetic sequences which hybridize to either controlling or transcribed segments of a receptor gene of the present invention. Three segments of particular interest are the controlling elements, both upstream and downstream, and segments encoding the DNA-binding segments and the hormone-binding segments. Methods applicable to such screening are analogous to those generally used in hybridization or affinity labeling.

Nucleic acid probes will often be labeled using radioactive or non-radioactive labels, many of which are listed in the section on polypeptide labeling. Standard procedures for nucleic acid labeling are described, e.g., in Sambrook et al. (1989); and Ausubel et al. (1987 and supplements).

Insect steroid receptor superfamily member polypeptides

A polypeptide sequence of the ecdysone receptor is represented in Table 2. Other insect steroid receptor superfamily member polypeptide sequences are set forth in Tables 1 and 3. Preferred nucleic acid sequences of the cDNAs encoding these insect steroid receptor superfamily member polypeptides are also provided in the corresponding tables. Other nucleic acids will be used to encode the proteins, making use of the degeneracy or non-universality of the genetic code.

As used herein, the term "substantially pure" describes a protein or other material, e.g., nucleic

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acid, which has been separated from its native
contaminants. Typically, a monomeric protein is
substantially pure when at least about 60 to 75% of a
sample exhibits a single polypeptide backbone. Minor

5   variants or chemical modifications typically share the
same polypeptide sequence. Usually a substantially pure
protein will comprise over about 85 to 90% of a protein
sample, and preferably will be over about 99% pure.
Normally, purity is measured on a polyacrylamide gel,

10   with homogeneity determined by staining. Alternatively,
for certain purposes high resolution will be necessary
and HPLC or a similar means for purification will be
used. For most purposes, a simple chromatography column
or polyacrylamide gel will be used to determine purity.

15   The term "substantially free of naturally-associated
insect cell components" describes a protein or other
material, e.g., nucleic acid, which is separated from the
native contaminants which accompany it in its natural
insect cell state. Thus, a protein which is chemically

20   synthesized or synthesized in a cellular system different
from the insect cell from which it naturally originates
will be free from its naturally-associated insect cell
components. The term is used to describe insect steroid
receptor superfamily members and nucleic acids which have

25   been synthesized in mammalian cells or plant cells, E.
coli and other procaryotes.

The present invention also provides for analogues of
the insect steroid receptor superfamily members. Such
analogues include both modifications to a polypeptide

30   backbone, e.g., insertions and deletions, genetic
variants, and mutants of the polypeptides. Modifications
include chemical derivatizations of polypeptides, such as
acetylations, carboxylations and the like. They also
include glycosylation modifications and processing

35   variants of a typical polypeptide. These processing
steps specifically include enzymatic modifications, such
as ubiquitinylation. See, e.g., Hershko and Ciechanover

(1982), "Mechanisms of Intracellular Protein Breakdown,"
Ann. Rev. Bioch., 51:335-364.

Other analogues include genetic variants, both
natural and induced. Induced mutants are derived from
various techniques, e.g., random mutagenesis using
reagents such as irradiation or exposure to EMS, or
engineered changes using site-specific mutagenesis
techniques or other techniques of modern molecular
biology. See, e.g., Sambrook et al. (1989) Molecular
Cloning: A Laboratory Manual (2d ed.), CSH Press; and
Ausubel et al. (1987 and supplements) Current Protocols
in Molecular Biology, Greene/Wiley, New York, each of
which is hereby incorporated herein by reference.

As described above, the DNA-binding zinc fingers
segment of a receptor shows high specificity of
recognition of specific target DNA sequences. An
understanding of the DNA-protein binding interactions
provides for the modification in a rational manner of
either DNA or protein characteristics, or both, to effect
specificity of binding for modulation of enhancer
activity. More importantly, isolation of genes for new
members of the insect steroid receptor superfamily allows
their use to produce the receptor polypeptides and to
isolate new controlling elements. By using the DNA-
binding domains, as described above, controlling elements
which are responsive to the ligands bound by the
corresponding superfamily members are identified and
isolated. This procedure shall yield a variety of
controlling elements responsive to ligands. By the
methods described above, the ligands for any particular
member of the insect steroid receptor superfamily will be
identified.

The controlling elements typically are enhancers,
but also include silencers or various other types of
ligand-responsive elements. They usually operate over
large distances, but will typically be within about
50 kb, usually within about 35 kb, more usually within

about 20 kb and preferably within about 7 kb of the genes
that these elements regulate.

Polypeptide fragments and fusions

Besides substantially full-length polypeptides, the
present invention provides for biologically active
fragments of the polypeptides. Significant biological
activities include ligand-binding, DNA binding,
immunological activity and other biological activities
characteristic of steroid receptor superfamily members.
Immunological activities include both immunogenic
function in a target immune system, as well as sharing of
immunological epitopes for binding, serving as either a
competitor or substitute antigen for a steroid receptor
epitope.

For example, ligand-binding or DNA-binding domains
from different polypeptides will be exchanged to form
different or new fusion polypeptides or fragments. Thus,
new chimaeric polypeptides exhibiting new combinations of
specificities result from the functional linkage of
ligand-binding specificities to DNA-binding domains.
This is extremely useful in the design of inducible
expression systems.

For immunological purposes, immunogens will
sometimes be produced from tandemly repeated polypeptide
segments, thereby producing highly antigenic proteins.
Alternatively, such polypeptides will serve as highly
efficient competitors for specific binding. Production
of antibodies to insect steroid receptor superfamily
members is described below.

The present invention also provides for other
polypeptides comprising fragments of steroid receptor
superfamily members. Fusion polypeptides between the
steroid receptor segments and other homologous or
heterologous proteins are provided, e.g., polypeptide
comprising contiguous peptide sequences from different
proteins. Homologous polypeptides will often be fusions

between different steroid receptor superfamily members,
resulting in, for instance, a hybrid protein exhibiting
ligand specificity of one member and DNA-binding
specificity of another. Likewise, heterologous fusions,
derived from different polypeptides, will be constructed
which would exhibit a combination of properties or
activities of the parental proteins. Typical examples
are fusions of a reporter polypeptide, e.g., luciferase,
with another domain of a receptor, e.g., a DNA-binding
domain, so that the presence or location of a desired
ligand is easily determined. See, e.g., Dull et al.,
U.S. No. 4,859,609, which is hereby incorporated herein
by reference. Other typical gene fusion partners include
"zinc finger" segment swapping between DNA-binding
proteins, bacterial ß-galactosidase, trpE Protein A,
ß-lactamase, alpha amylase, alcohol dehydrogenase, and
yeast alpha mating factor. See, e.g., Godowski et al.
(1988), Science 241:812-816; and Experimental section
below.

Insect steroid receptor superfamily member expression
        With the sequence of the receptor polypeptides and
the recombinant DNA sequences encoding them, large
quantities of members of the insect steroid receptor
superfamily will be prepared. By the appropriate
expression of vectors in cells, high efficiency protein
production will be achieved. Thereafter, standard
protein purification methods are available, such as
ammonium sulfate precipitation, column chromatography,
electrophoresis, centrifugation, crystallization, and
others. See, e.g., Deutscher (1990) "Guide to Protein
Purification" Methods in Enzymology, vol 182 and others;
and Ausubel et al. (1987 and supplements) Current
Protocols in Molecular Biology, for techniques typically
used for protein purification. Alternatively, in some
embodiments high efficiency of production is unnecessary,
but the presence of a known inducing protein within a

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carefully engineered expression system is quite valuable.
For instance, a combination of: (1) a ligand-responsive
enhancer of this type operably linked to (2) a desired
gene sequence with (3) the corresponding insect steroid
receptor superfamily member will be placed together in an
expression system provides a specifically inducible
expression system. The desired gene sequence will encode
a protein of interest, and the corresponding steroid
receptor member will often be the ecdysone receptor.
Typically, the expression system will be a cell, but _in_
vitro expression systems will also be constructed.

The desired genes will be inserted into any of a
wide selection of expression vectors. The selection of
an appropriate vector and cell line depends upon the
constraints of the desired product. Typical expression
vectors are described in Sambrook et al. (1989) and
Ausubel et al. (1987 and supplements). Suitable cell
lines are available from a depository, such as the ATCC.
See, ATCC Catalogue of Cell Lines and Hybridomas (6th
ed.) (1988); ATCC Cell Lines, Viruses, and Antisera, each
of which is hereby incorporated herein by reference. The
vectors are introduced to the desired cells by standard
transformation or transfection procedures as described,
for instance, in Sambrook et al. (1989).

Fusion proteins will typically be made by either
recombinant nucleic acid methods or by synthetic
polypeptide methods. Techniques for nucleic acid
manipulation are described generally, for example, in
Sambrook et al. (1989), _Molecular Cloning: A Laboratory_
Manual (2d ed.), Vols. 1-3, Cold Spring Harbor
Laboratory, which are incorporated herein by reference.
Techniques for synthesis of polypeptides are described,
for example, in Merrifield, _J. Amer. Chem. Soc._ 85:2149-
2156 (1963).

The recombinant nucleic acid sequences used to
produce fusion proteins of the present invention will
typically be derived from natural or synthetic sequences.

Many natural gene sequences are obtainable from various cDNA or from genomic libraries using appropriate probes. See, GenBank™, National Institutes of Health. Typical probes for steroid receptors are selected from the sequences of Tables 1, 2 or 3 in accordance with standard procedures. The phosphoramidite method described by Beaucage and Carruthers, Tetra. Letts. 22:1859-1862 (1981) will produce suitable synthetic DNA fragments. A double stranded fragment is then obtainable either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

With the isolated steroid receptor genes, segments of the transcribed segments are available as probes for isolating homologous sequences usually from different sources, e.g., different animals. By selection of the segment used as a probe, particular functionally associated segments will be isolated. Thus, for example, other nucleic acid segments encoding either ligand-binding or DNA-binding domains of new receptors will be isolated. Alternatively, by using steroid-responsive controlling elements as a probe, new steroid-responsive elements will be isolated, along with the associated segment of DNA whose expression is regulated. This method allows for the isolation of ligand-responsive genes, many of which are, themselves, also members of the insect steroid receptor superfamily.

The natural or synthetic DNA fragments coding for a desired steroid receptor fragment will be incorporated into DNA constructs capable of introduction to and expression in an in vitro cell culture. Usually the DNA constructs will be suitable for replication in a unicellular host, such as yeast or bacteria, but alternatively are intended for introduction to, with or without integration into the genome, cultured mammalian or plant or other eucaryotic cell lines. DNA constructs

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prepared for introduction into bacteria or yeast will typically include a replication system recognized by the host, the intended DNA fragment encoding the desired receptor polypeptide, transcription and translational initiation regulatory sequences operably linked to the polypeptide encoding segment and transcriptional and translational termination regulatory sequences operably linked to the polypeptide encoding segment. The transcriptional regulatory sequences will typically include a heterologous enhancer or promoter which is recognized by the host. The selection of an appropriate promoter will depend upon the host, but promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known. _See_, Sambrook et al. (1989). Conveniently available expression vectors which include the replication system and transcriptional and translational regulatory sequences together with the insertion site for the steroid receptor DNA sequence will generally be employed. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al. (1989); _see also_, Metzger et al. (1988), _Nature_ 334:31-36.

Genetic constructs

The DNA segments encoding the members of the insect steroid receptor superfamily will typically be utilized in a plasmid vector. In one embodiment an expression control DNA sequence is operably linked to the insect steroid receptor superfamily member coding sequences for expression of the insect steroid receptor superfamily member alone. In a second embodiment an insect steroid receptor superfamily member provides the capability to express another protein in response to the presence of an insect steroid receptor ligand. This latter embodiment is separately described below. The expression control sequences will commonly include eukaryotic enhancer or promoter systems in vectors capable of transforming or

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transfecting eucaryotic host cells. Once the vector has
been introduced into the appropriate host, the host,
depending on the use, will be maintained under conditions
suitable for high level expression of the nucleotide
5   sequences.

Steroid-responsive expression of selected genes
        For steroid-responsive expression of other genes,
the steroid receptor gene will typically be cotransformed
10   with a recombinant construct comprising a desired gene
for expression operably linked to the steroid-responsive
enhancer or promoter element. In this use, a single
expression system will typically comprise a combination
of (1) a controlling element responsive to a ligand of an
15   insect steroid receptor superfamily member, (2) a desired
gene for expression, operably linked to the controlling
element, and (3) an insect steroid receptor superfamily
member which can bind to the controlling element.
Usually, this system will be employed within a cell, but
20   an in vitro system is also possible. The insect steroid
receptor superfamily member will typically be provided by
expression of a nucleic acid encoding it, though it need
not be expressed at high levels. Thus, in one preferred
embodiment, the system will be achieved through
25   cotransformation of a cell with both the regulatable
construct and another segment encoding the insect steroid
receptor superfamily member. Usually, the controlling
element will be an enhancer element, but some elements
work to repress expression. In this embodiment, the
30   ligand for the insect steroid receptor superfamily member
will be provided or withheld as appropriate for the
desired expression properties.
        A particularly useful genetic construct comprises an
alcohol dehydrogenase promoter operably linked to an
35   easily assayable reporter gene, e.g., β-galactosidase.
In a preferred embodiment of this construct, a
multiplicity of copies of the insect steroid receptor

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superfamily member is used. For example, operable linkage of controlling elements responsive to insect steroid receptor superfamily members, e,g., EcR, DHR3, E75A and E75B, to the alcohol dehydrogenase (ADH) promoter, or others as described above, and protein coding sequences for a particular reporter protein, as described above, leads to steroid-responsive expression of ß-galactosidase. Such a system provides highly sensitive detection of expression in response to ligand binding, allowing for detection of a productive ligand-receptor interaction.

DNA sequences will normally be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

E. coli is one procaryotic host useful for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilis, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species.

Other eucaryotic cells will often be used, including yeast cells, insect tissue culture cells, avian cells, or the like. Preferably, mammalian tissue cell culture will be used to produce the inducible polypeptides of the present invention (see, Winnacker, From Genes to Clones, VCH Publishers, N.Y. (1987), which is incorporated herein by reference). Mammalian cells are preferred cells in which to use the insect steroid receptor superfamily member ligand-responsive gene constructs, because they naturally lack the molecules which confer responses to

the ligands for insect steroid receptor superfamily members.

Mammalian cells are preferred because they are insensitive to many ligands for insect steroid receptor superfamily member. Thus, exposure of these cells to the ligands of the insect steroid receptor superfamily members typically will have negligible physiological or other effects on the cells, or on a whole organism. Therefore, cells can grow and express the desired product, substantially unaffected by the presence of the ligand itself. The ligand will function to cause response either in the positive or negative direction. For example, it is often desirable to grow cells to high density before expression. In a positive induction system, the inducing ligand would be added upon reaching high cell density, but since the ligand itself is benign to the cells, the only physiological imbalances result from the expression, e.g., the product, itself. Alternatively, in a negative repression system, the ligand is supplied until the cells reach a high density. Upon reaching a high density, the ligand would be removed. Introduction of these cells into a whole organism, e.g., a plant or animal, will provide the products of expression to that organism. In this circumstance, the natural insensitivity of cells to the ligands will also be advantageous.

Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferably, the enhancers or promoters will be those naturally associated with genes encoding the steroid receptors, although it will be understood that in many cases others will be equally or more appropriate. Other preferred expression control sequences are enhancers or promoters derived from

viruses, such as SV40, Adenovirus, Bovine Papilloma Virus, and the like.

Similarly, preferred promoters are those found naturally in immunoglobulin-producing cells (see, U.S. Patent No. 4,663,281, which is incorporated herein by reference), but SV40, polyoma virus, cytomegalovirus (human or murine) and the LTR from various retroviruses (such as murine leukemia virus, murine or Rous sarcoma virus and HIV) are also available. See, Enhancers and Eukaryotic Gene Expression, Cold Spring Harbor Press, N.Y., 1983, which is incorporated herein by reference.

The vectors containing the DNA segments of interest (e.g., the steroid receptor gene, the recombinant steroid-responsive gene, or both) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for procaryotic cells, whereas calcium phosphate treatment is often used for other cellular hosts. See, generally, Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual (2d ed.), Cold Spring Harbor Press; Ausubel et al. (1987 and supplements) Cement Protocls in Molecular Biology, Greene/Wiley, New York; and Potrykus (1990) "Gene Transfer to Cereals: An Assessment," Bio/Technology 8:535-542, each of which is incorporated herein by reference. Other transformation techniques include electroporation, DEAE-dextran, microprojectile bombardment, lipofection, microinjection, and others. The term "transformed cell" is meant to also include the progeny of a transformed cell.

As with the purified polypeptides, the nucleic acid segments associated with the ligand-binding segment and the DNA-binding segment are particularly useful. These gene segments will be used as probes for screening for new genes exhibiting similar biological activities, though the controlling elements of these genes are of equal importance, as described below.

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Many types of proteins are preferentially produced in eucaryotic cell types because of abnormal processing or modification in other cell types. Thus, mammalian proteins are preferably expressed in mammalian cell cultures. Efficient expression of a desired protein is often achieved, as described above, by placing: (1) a desired protein encoding DNA sequence adjacent to controlling elements responsive to ligands for insect steroid receptor superfamily members and an appropriate promoter. Cyclic pulses of ligands in a cell culture may provide periods for cells to recover from effects of production of large amounts of exogenous protein. Upon recovery, the ligand will often be reinduced.

Additional steroid responsive gene elements have also been isolated, e.g., substantially purified, using the techniques of the present invention. Other genes adjacent to, and operably linked to, steroid responsive gene control elements are selectable by locating DNA segments to which steroid receptors specifically bind or by hybridization to homologous controlling elements. For example, other steroid responsive genes have been isolated. Many of the genes which are ligand-responsive will also be new members of the insect steroid receptor superfamily.

Having provided for the substantially pure polypeptides, biologically active fragments thereof and recombinant nucleic acids comprising genes for them, the present invention also provides cells comprising each of them. By appropriate introduction techniques well known in the field, cells comprising them will be produced. See, e.g., Sambrook et al. (1989).

In particular, cells comprising the steroid responsive controlling elements are provided, and operable linkage of standard protein encoding segments to said controlling elements produce steroid responsive systems for gene expression. Cells so produced will often be part of, or be introduced into, intact

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organisms, for example, plants, insects (including
caterpillars and larvae), and higher animals, e.g.,
mammals. This provides for regulatable expression of
desired genes where the regulating ligand has no other
effects on the cells because the cells otherwise lack the
receptors and responsive genes. For example, plants will
be induced to fruit at desired times by administration of
the appropriate ligand, or animals will be ligand-
responsive in production of particular products. And, in
fact, biochemical deficiencies may be overcome by ligand-
responsive expression of cells introduced into an intact
organism which, itself, also otherwise lacks genes
responsive to the presence of such a ligand. Multiple
repeats of the control elements will lead, often, to at
least additive or synergistic control. Cells containing
these expression systems will be used in gene therapy
procedures, including in humans.

Once a sufficient quantity of the desired steroid
receptor polypeptide has been obtained, the protein is
useful for many purposes. A typical use is the
production of antibodies specific for binding to steroid
receptors. These antibodies, either polyclonal or
monoclonal, will be produced by available *in vitro* or *in
vivo* techniques.

For production of polyclonal antibodies, an
appropriate target immune system is selected, typically a
mouse or rabbit. The substantially purified antigen is
presented to the immune system in a fashion determined by
methods appropriate for the animal and other parameters
well known to immunologists. Typical sites for injection
are in the footpads, intramuscularly, intraperitoneally,
or intradermally. Of course, another species will often
be substituted for a mouse or rabbit.

An immunological response is usually assayed with an
immunoassay. Normally such immunoassays involve some
purification of a source of antigen, for example,
produced by the same cells and in the same fashion as the

antigen was produced. The immunoassay will generally be
a radioimmunoassay, an enzyme-linked assay (ELISA), a
fluorescent assay, or any of many other choices, most of
which are functionally equivalent but each will exhibit
advantages under specific conditions.

Monoclonal antibodies with affinities of 10^8 M^{-1}
preferably 10^9 to 10^{10}, or stronger will typically be
made by standard procedures as described, e.g., in Harlow
and Lane (1988), Antibodies: A Laboratory Manual, Cold
Spring Harbor Laboratory; or Goding (1986), Monoclonal
Antibodies: Principles and Practice (2d ed) Academic
Press, New York, which are hereby incorporated herein by
reference. Briefly, appropriate animals will be selected
and the desired immunization protocol followed. After
the appropriate period of time, the spleens of such
animals are excised and individual spleen cells fused,
typically, to immortalized myeloma cells under
appropriate selection conditions. Thereafter the cells
are clonally separated and the supernatants of each clone
are tested for their production of an appropriate
antibody specific for the desired region of the antigen.

Other suitable techniques involve in vitro exposure
of lymphocytes to the antigenic polypeptides or
alternatively to selection of libraries of antibodies in
phage or similar vectors. See, Huse et al., (1989)
"Generation of a Large Combinatorial Library of the
Immunoglobulin Repertoire in Phage Lambda," Science
246:1275-1281, hereby incorporated herein by reference.

The polypeptides and antibodies of the present
invention will be used with or without modification.
Frequently, the polypeptides and antibodies will be
labeled by joining, either covalently or non-covalently,
a substance which provides for a detectable signal. A
wide variety of labels and conjugation techniques are
known and are reported extensively in both the scientific
and patent literature. Suitable labels include
radionuclides, enzymes, substrates, cofactors,

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inhibitors, fluorescens, chemiluminescers, magnetic
particles and the like. Patents, teaching the use of
such labels include U.S. Patent Nos. 3,817,837;
3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149;
5   and 4,366,241. Also, recombinant, chimeric, or
humanized, immunoglobulins will be produced, see, e.g.,
Cabilly, U.S. Patent No. 4,816,567; Jones et al., 1986,
Nature 321, 522-526; and published UK patent application
No. 8707252; each of which is hereby incorporated herein
10   by reference.

        Another use of purified receptor polypeptides is for
determination of the structural and biosynthetic aspects
of the polypeptides. Structural studies of interactions
of the ligand-binding domains with selected ligands are
15   performed by various methods. The preferred method for
structural determination is X-ray crystallography but may
include various other forms of spectroscopy or
chromatography. See, e.g., Connolly, M.L., J. Appl.
Crystall., 16:548 (1983); Connolly, M.L., Science 221:709
20   (1983); and Blundell and Johnson (1976) Protein
Crystallography, Academic Press, New York; each of which
is hereby incorporated herein by reference. For example,
the structure of the interaction between hormone ligand
and hormone-binding segments is determined to high
25   resolution. From this information, minor substitutions
or modifications to either or both of the ligand and
ligand-binding segment are made based upon, e.g., the
contact regions between the two. This information
enables the generation of modified interactions between a
30   ligand and its binding segment to either increase or
decrease affinity of binding and perhaps increase or
decrease response to binding. Likewise, the interaction
between the zinc finger DNA-binding segments with the
specific nucleic acid-binding sequence will be similarly
35   modified.

        As a separate and additional approach, isolated
ligand-binding polypeptide domains will be utilized to

screen for new ligands. Binding assays will be
developed, analogous, e.g., to immunoassays. This
procedure permits screening for new agonists or
antagonists of a particular steroid receptor. Isolated
DNA-binding segments will be used to screen for new DNA
sequences which will specifically bind to a particular
receptor-binding segment. Typically, these receptor-
specific binding sites will be controlling elements for
steroid responsive genes. Thus, having isolated these
DNA-binding sequences, genes which are responsive to the
binding of a given receptor can be isolated. This
provides a method for isolating genes which are
responsive to induction or inhibition by a given hormone
receptor.

In another aspect of the present invention, means
for disrupting insect development are provided where new
ligand agonists or antagonists are discovered. These
compounds are prime candidates as agonists or antagonists
to interfere with normal insect development. By
application of new steroid analogues of ligands for
insect steroid receptor superfamily members, it is
possible to modify the normal temporal sequence of
developmental events. For example, accelerating insect
development will minimize generation time. This will be
very important in circumstances where large numbers of
insects are desired finally, for instance, in producing
sterile males in Mediterranean fly infestations.
Alternatively, it is useful to slow development in some
pest infestations, such that the insects reach
destructive stages of development only after commercial
crops have passed sensitive stages.

In another commercial application, ligands
discovered by methods provided by the present invention
will be used in the silk-production industry. Here, the
silkworms are artificially maintained in a silk-producing
larvae stage, thereby being silk productive for extended
time periods. The development of larvae will also be

susceptible to acceleration to silk-production in a
shorter time period than than naturally.

Other analogues of ligands for insect steroid
receptor superfamily members will be selected which, upon
application, will completely disrupt normal development
and, preferably, lead to a lethal result. Slightly
modified natural substances will often have greater
specificity of action and much higher activities,
allowing for lower levels of application. For example,
more lipophilic ligands are more readily absorbed
directly into the insect surface or cuticle. Thus,
extremely low concentrations of natural ligands should be
effective in controlling pests. Furthermore, many of
these ligands are likely to be relatively easily
manufactured, taking advantage of enzymatic production
methods. New ligands for insect steroid receptor
superfamily members will sometimes be more species
specific or will exhibit particularly useful
characteristics, for example, being lethal to specific
harmful insects. The greater specificity of the hormones
will allow avoidance of the use of non-specific
pesticides possessing undesired deleterious ecological
side effects, e.g., pesticide residue accumulation in
food, often having deleterious effects on humans.
Furthermore, compounds having structures closely
analogous to natural compounds should be susceptible to
natural mechanisms of biological degradation.

Another aspect of the present invention provides for
the isolation or design of new gene segments which are
responsive to ligands for insect steroid receptor
superfamily members. For example, use of the nucleic
acids to screen for homologous sequences by standard
techniques will provide genes having similar structural
features. Similarly arranged intron structures will
typically be characteristic of larger superfamily
categories. The preferred domains for screening will be
the ligand-binding or DNA-binding segments, although the

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DNA segments which are recognized by the DNA-binding domains, i.e., the controlling elements, will also be of particular interest. Screening for new controlling elements will usually take advantage of known similarities, e.g., sequence homology to other known elements, or homology to the DNA zinc finger-binding domains of other receptors. Receptors and genes important in the general developmental sequence of expression will be discovered. Using this set of developmentally regulated genes will allow selection of particular molecules which are responsible for controlling expression of developmentally regulated genes.

Kits for the determination of expression levels of the nucleic acids and proteins provided herein are made available. Typically, the kit will have at least one compartment which contains a reagent which specifically binds to the desired target molecule, e.g., ligand analogues, receptors, or nucleic acids. These reagents will be used in techniques for assays, e.g., using methods typically used in screening protocols. See, e.g., Sambrook et al. (1989) and Ausubel et al. (1987 and supplements).

The following experimental section is offered by way of example and not by limitation.

EXPERIMENTAL

EXAMPLE I

CLONING STRUCTURE AND EXPRESSION OF THE DROSOPHILA E75 GENE THAT ENCODES TWO MEMBERS OF THE STEROID RECEPTOR SUPERFAMILY.

The following experiments demonstrate that the E75 gene encodes two members of the steroid receptor superfamily. The proteins it encodes share amino acid sequence homology with the conserved DNA-binding and ligand-binding domains of this superfamily. The E75 gene is ecdysone-inducible, and it occupies and causes the

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ecdysone-inducible early puff at the 75B locus in the
Drosophila polytene chromosome.

A.   Cloning of Genomic DNA Encompassing the
      Ecdysone-Inducible 75B Puff Locus

We have used the method of chromosomal walking
(Bender, W., P. Spierer, and D. S. Hogness, 1983.
Chromosomal walking and jumping to isolate DNA from the
Ace and rosy loci and the Bithorax complex in Drosophila
melanogaster. J. Mol. Biol. 168:17-33) to isolate the
genomic DNA encompassing the 75B puff region. The
starting point for the walk was a genomic clone,
designated λ8253, which had been localized by in situ
hybridization to the proximal end of 75B. Isolated
restriction fragments of λ8253 were used to screen a
library of genomic DNA from the Canton S (C^S) strain of
D. melanogaster. See (Maniatis, T., R. C. Hardison, E.
Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and
A. Efstradiatis, 1978, "The isolation of structural genes
from libraries of eucaryotic DNA." Cell 15:687-701).
Genomic clones λcDm3504 and λcDm3505 were isolated by
homology to λ8253.

The walk was then extended in both directions until
~100 kb of genomic DNA had been isolated, and the
orientation of the walk was determined by in situ
hybridization of the terminal segments to polytene
chromosomes. Thereafter, the walk was extended in the
rightward direction on the molecular map, or distally
relative to the centromere. The 350 kb of genomic DNA
encompassed by the walk corresponds to the chromosomal
region between bands 75A6-7 and 75B11-13, as determined
by in situ hybridization. This region includes the 75B
puff, which appears to initiate by simultaneous
decondensation of chromosomal bands 75B3-5 and then
spreads to surrounding bands.

Methods

Genomic DNA libraries

Canton S genomic DNAs were isolated from a library of sheared, EcoRI-linkered Canton S DNA cloned into the Charon 4 λ phage vector. See (Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and A. Efstradiatis, 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell 15:687-701). O^r genomic DNAs were isolated from a library of sheared DNA, GC-tailed into the sep6 λ vector. See (Meyerowitz, F. M., and D. S. Hogness, 1982. "Molecular organization of a Drosophila puff site that responds to ecdysone." Cell 28:165-176). One step in the chromosomal walk was taken using a cosmid library of SauIIIA partially digested O^r DNA cloned into the cosmid p14B1 by the method of Ish-Horowicz and Burke (Ish-Horowicz, D., and J. F. Burke, 1982. Rapid and efficient cosmid cloning. Nucleic Acids Res. 9:2989-2998).

In situ hybridization

In situ hybridization to polytene chromosomes was carried out with DNA probes that were nick-translated in the presence of ^{3}H-labeled TTP (NEN), as described by Bonner and Pardue (Bonner, J. J., and M. L. Pardue, 1976. Ecdysone-stimulated RNA synthesis in imaginal discs of Drosophila melanogaster. Assay by in situ hybridization. Chromosoma 58:87-99), with the following modifications: Heat and RNAase treatments of the slides were omitted, and hybridization and washing were at 63°C in 2XSSPE for 18 and 2 hours, respectively.

B. Identification of a 50 kb Region of Cloned Genomic DNA that Contains Sequences Homologous to Ecdysone-induced Transcripts

Restriction fragments of the above genomic clones were tested for their ability to hybridize with each of two cDNA probes, one derived from the RNA in ecdysone-induced cells, and the other from the RNA in noninduced cells. Two differential screens were carried out. In the first, genomic DNA covering the entire 350 kb walk

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was examined with cDNA probes synthesized with reverse transcriptase from an oligo(dT) primer annealed to poly(A)+ RNA. The poly(A)+ RNA was prepared from total inner tissues that were mass-isolated from late third

5   instar larvae and incubated in the presence of ecdysone plus cycloheximide, or cycloheximide alone. (See Methods, below. Cycloheximide was included because higher levels of ecdysone-induced transcripts accumulate in its presence.)

10   Each of the ^{32}P-labeled cDNA probes made from these two poly(A)+ RNAs was applied to one of two duplicate Southern blots that contained, in addition to the genomic fragments from the walk, a control DNA consisting of sequences from the ribosomal protein 49 gene (O'Connell,

15   P., and M. Rosbash, 1984. Sequence, structure and codon preference of the Drosophila ribosomal protein 49 gene. Nucleic Acids Res. 12:5495-5513), which was used to normalize the hybridization intensities of the duplicate blots. This screen revealed sequences specific to

20   ecdysone-induced RNAs only within the λcDm3522 genomic clone that is centered at approximately +220 kb on the molecular map.

         Because the above probes will preferentially detect sequences near the 3' termini of the RNAs, particularly

25   in the case of long transcripts, a second differential screen was carried out with cDNA probes primed with random hexamers (see Methods, below). This screen, which was restricted to the 135 kb of genomic DNA between +105 kb and +240 kb, revealed ecdysone-inducible sequences in

30   fragments spread out over an ~50 kb region between +170 kb and +220 kb. This region represents the E75 gene.

Methods

Organ culture and RNA isolation

35   Late third instar O^r larvae were harvested, washed in 0.7% NaCl, resuspended in Robb's phosphate-buffered saline (PBS) (Robb, J. A., 1968. Maintenance of imaginal

discs of <u>Drosophila melanogaster</u> in chemically defined
media. <u>J. Cell. Biol.</u> 41:876-885), preaerated with a
blender, and passed through a set of rollers to extrude
the organs. This "grindate" was filtered through a
coarse Nitex screen to remove carcasses, and settled five
times (3-5 minutes per settling) by gravity to remove
floating and microscopic debris. Isolated tissues
(primarily salivary glands, imaginal discs, gut, and
Malphigian tubules) were cultured at 25°C in plastic
petri dishes in aerated Robb's PBS. β-ecdysone (Sigma)
(0.2 μl/ml of 10 mg/ml) in ethanol and/or cycloheximide
(2 μl/ml of 35 mM) in water was added to the appropriate
cultures. Incubations in the presence of cycloheximide
were for ~8 hours. Isolated tissues were homogenized in
10 volumes of 6 M guanidine-HCl/0.6 M sodium acetate
(pH 5.2), centrifuged at 5000 g for 10 minutes to remove
debris, and layered onto a 5.7 M CaCl shelf, as described
previously (Chirgwin, J. M., A. E. Przbyla,
R. J. MacDonald, and W. J. Rutter, 1979. Isolation of
biologically active ribonucleic acid from sources
enriched in ribonuclease. <u>Biochemistry</u> 18:5294-5299).
Poly(A)+ RNA was purified by oligo(dT) chromatography.

<u>Southern blot analysis</u>

Southern blots were performed on nitrocellulose, as
described previously (Segraves, W. A., C. Louis, S.
Tsubota, P. Schedl, J. M. Rawls, and B. P. Jarry, 1984.
The rudimentary locus of <u>Drosophila melanogaster</u>. <u>J.
Mol. Biol.</u> 175:1-17). cDNA probes were prepared by
reverse transcription (AMV reverse transcriptase;
Seikagaku) of 2 μg of poly(A)+ RNA with 700 ng of
oligo(dT)[12-16] (Collaborative Research) or 15 μg of random
hexamers (Pharmacia) in a 20 μl reaction mixture
containing 80 mM Tris Cl (pH 8.3 at 42°C), 10 mM MgCl$_2$,
100 mM KCl, 0.4 mM DTT, 0.25 mM each of dATP, dGTP, and
dTTP, and 100 μCi of [^{32}P]dCTP (800 Ci/mole; Amersham).
After incubation at 37°C for 45 minutes, 80 μl of 10 mM

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EDTA and 2 µl of 5 N NaOH were added before incubation at
70°C for 10 minutes to denature the products and
hydrolyze the RNA. After the addition of 10 µl of 1 M
Tris-Cl (pH 7.5) and 5 µl of 1 N HCl, unincorporated
label was removed by chromatography on Biogel P60.

C.   The E75 Gene Contains Two Overlapping
     Transcription Units: E75A and E75B

Northern blot analysis of ecdysone-induced and
noninduced RNAs, prepared as described above and
hybridized with strand-specific DNA probes derived from
cloned restriction fragments in the 60 kb region (+166 to
+226 kb) containing the E75 gene, demonstrated that this
gene produces two classes of ecdysone-inducible mRNAs,
both derived from rightward transcription. The E75A
class of mRNAs hybridized with probes from both the 5'
(left) and 3' (right) ends of the 50 kb E75 gene. The
E75B class hybridized only with probes from the 3'-
proximal 20 kb of the gene. These results suggest that
the A and B classes of ecdysone-inducible RNAs are
initiated by different promoters, located about 30 kb
apart, and that the two transcription units defined by
these promoters overlap in the region downstream from the
B promoter.

This suggestion was confirmed by analysis of the
structure of cloned cDNAs from the E75A and E75B mRNAs.
Approximately 10^6 clones from an early pupal cDNA library
(Poole, S. J., L. M. Kauvar, B. Drees, and T. Kornberg,
1985. The engrailed locus of Drosophila: Structural
analysis of an embryonic transcript. Cell 40:37-40) were
screened at low resolution with genomic DNA probes from
the E75 gene region. The 116 cDNA clones identified by
this screen were analyzed by restriction digestion and
hybridization to a panel of probes derived from the 60 kb
(+166 to +226 kb) region. One of the clones, λDm4925,
was thereby selected as a representative of the E75A

class of mRNAs, and another, λDm4745, as a representative
of the E75B mRNA class.

The genomic regions homologous to these two cDNA
clones were further localized by Southern blot analysis,
and the nucleotide sequence of these regions and of both
cDNA clones was determined. These sequences are given in
Table 1, along with those derived from 5' and 3' terminal
sequence determinations for each transcription unit.
These data demonstrate that the 50 kb E75A transcription
unit consists of six exons, labeled in 5' to 3' order:
A0, A1, 2, 3, 4 and 5, of which exons A0 and A1 are
specific to this unit, while the remaining four are
shared with the 20 kb E75B transcription unit.
Similarly, the E75B unit contains a specific exon,
labeled B1, at its 5' end, which is located just upstream
of the shared exon 2. Thus, the E75 gene consists of two
transcription units, of which the shorter E75B unit
occupies the 3' proximal 20 kb of the longer E75A unit.

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Table 1. Sequences of the E75 exons and
flanking DNA. The sequence is that of the C^S genomic
DNA, which was identical to that of the cDNAs, except for
the T→G change indicated at position +2691. This change
would convert a leucine to an arginine in the protein
sequences. The Dm4925 cDNA extends from just 5' of the
EcoRV site at +939 to +4267 in A. The Dm4745 cDNA
extends from +804 in B to a point near the HindIII site
at +4246 in A. (A) The E75 A exons and flanking DNA. The
sequences of the A0, A1, and common exons 2-5 are
interrupted by intron sequences (lowercase), which are
limited to those near the splice sites and are in
agreement with consensus sequences for donor (5') and
acceptor (3') splice sites. Negative numbers at the
right end of each line refer to the number of base pairs
upstream of the E75 A initiation site, positive numbers
refer to positions in the E75 A mRNAs, continuing into
the 3' flanking DNA. Numbers at the left end of each
line refer to amino acid residues in the E75 A protein.
The underlined 14 bp sequence at -159 to -172 exhibits a
13/14 bp match to a sequence (CGTAGCGGGTCTC) found 47 bp
upstream of the ecdysone-inducible E74 A transcription
unit responsible for the early puff at 74EF. This
sequence represents the proximal part of a 19 bp sequence
in the E74 A promoter that binds the protein encoded by
the D. melanogaster zeste gene. Another underlined
sequence in the E75 A promoter at -74 to -82 is also
found in the E75 B promoter, where it is part of a
tandemly repeated octanucleotide (GAGAGAGC) located at
-106 to -121 in B. This repeat matches the consensus
sequence for the binding sites of the GAGA transcription
factor which also binds to the E74 A promoter. Other
underlined sequences represent, at -27 to -33, the best
match to the TATA box consensus at an appropriate
position, three AUG codons that are closely followed by
in-frame stop codons in the 5'-leader sequence of the E75
mRNAs, and alternative polyadenylation-cleavage signals
at 4591 and 5365 that are used by both E75 A and E75B
mRNAs. (B) The B1 exon and its 5'-flanking DNA. The
numbering at the right and left ends of the lines follows
the same convention as in A. Exons 2-5 shown in A are
also used in E75 B, but the amino acid residues and base
pair numbers shown in A must be increased by 157 and 375,
respectively, to apply to the E75 B protein and mRNA.
The first ten nucleotides of the 136-nucleotide E75
B-intron linking the B1 exon to Exon 2 are gtaggttag,
whereas the last ten are shown upstream of nucleotide
1178 in A. The underlined sequences represent, in order,
the region of homology to a sequence upstream of E75 A,
noted above, the best match to the TATA box consensus
at -21 to -27, and three AUG codons followed by in-frame
stop codons in the 5' leader of the E75 B mRNA.
Panels 1 and 2 are shown in detail in panels 3-8,
and 9-10, respectively.

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Methods
cDNA libraries

The λDm4925 and λDm4745 cDNAs were isolated from an
0ʳ early pupal cDNA library in λgt10 (Poole, S. J.,
L. M. Kauvar, B. Drees, and T. Kornberg, 1985. The
engrailed locus of Drosophila: structural analysis of an
embryonic transcript. Cell 40:37-40). The two cDNAs
(λDm4927 and λDm4928) that were used for 3'-end mapping
were isolated from an ecdysone-induced salivary gland
cDNA library in λ607 prepared by C. W. Jones. (Our
strain collection names for the cDNA clones used in these
studies are λfDm4925, λfDm4745, λeDm4927, and λeDm4928.)

Northern blot analysis

Probes to be used for Northern blots were cloned
into the vector pφX (from R. Mulligan), containing the
φX174 origin of replication cloned in between the HindIII
and BamHI sites of pBR322. This allowed the synthesis of
single-stranded probe DNA (Arai, K., N. Arai, J.
Schlomai, and A. Kornberg, 1980. Replication of duplex
DNA of phage φX174 reconstituted with purified enzymes.
Proc. Natl. Acad. Sci. 77:3322-3326), which was performed
by the incubation of supercoiled plasmid DNA with gene A
protein, rep and ssb proteins, and DNA polymerase III
holoenzyme in a reaction containing 20 mM Tris Cl
(pH 7.5), 80 μg/ml BSA, 4% glycerol, 20 mM DTT, 1 mM ATP,
16 mM concentrations of the three unlabeled
deoxynucleotides and 1.6 mM concentrations of the labeled
deoxynucleotide for 1 hour at 30°C. EDTA was then added
to 20 mM, SDS to 0.1%, and proteinase K to 50 μg/ml. The
reactions were digested for 30 minutes at 37°C, and
unincorporated label was removed by gel filtration.

S1 nuclease protection and primer extension analysis

Single-stranded probes, prepared as described above
by the φX in vitro replication system, were purified by
electrophoresis on low melting point agarose gels for use

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as S1 probes. All other probes were prepared by
extension of the -20, 17-mer sequencing primer (New
England Biolabs) on single-stranded M13mp (Messing, J.,
1983. New M13 vectors for cloning. Methods Enzymol.
101:20-78) or pEMBL (Dente, L., G. Cesareni, and R.
Cortes, 1983. pEMBL: A new family of single-stranded
plasmids. Nucleic Acids Res. 11:1645-1654) recombinant
templates using ^{32}P-labeled nucleotides, followed by
cleavage with the appropriate restriction enzyme and
purification of the probe on denaturing polyacrylamide
gels. Labeled probe (100,000-300,000 cpm) was incubated
with 1 μg of poly(A)+ RNA in a 5 μl reaction mixture
containing 5 μg of yeast tRNA, 0.4 M NaCl, 40 mM PIPES
(pH 6.8), and 1 mM EDTA at 60°C under oil. Reactions
were cooled and diluted 1:10 into either S1 digestion or
primer extension buffer. S1 nuclease digestions were
performed in 50 mM acetate buffer (Na), 400 mM NaCl, and
4 mM $ZnSO_4$ at 20°C for 1 hour with ~15 - 150 Vogt units of
S1 nuclease (Boehringer) per 50 μl reaction. Primer
extensions were performed at 42°C in 50 mM Tris Cl
(pH 8.3 at 42°C), 80 mM KCl, 2 mM DTT, 1 mM of dATP,
dCTP, dGTP, and dTTP, with 20 units of AMV reverse
transcriptase (Seikagaku) per 50 μl reaction. Reactions
were terminated by the addition of EDTA, tRNA carrier was
added to the S1 nuclease digestions, and samples were
ethanol-precipitated and either electrophoresed directly
on 5% or 6% denaturing polyacrylamide gels or glyoxalated
(McMaster, G. K., and G. C. Carmichael, 1977. Analysis
of single and double-stranded nucleic acids on
polyacrylamide and agarose gels by using glyoxal and
acridine orange. Proc. Natl. Acad. Sci. 74:4835-4838)
and electrophoresed on 1% agarose gels run in 10 mM
sodium phosphate buffer (pH 6.8).

DNA sequence analysis

The cDNA clones λDm4927 and λDm4928 were sequenced
by chemical degradation (Maxam, A. M., and W. Gilbert,

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1980. Sequencing end-labeled DNA with base-specific chemical cleavage. Methods Enzymol. 65:499-560). All other sequencing was performed using the dideoxynucleotide chain termination method (Sanger, F., A. R. Coulson, B. F. Barrell, A. J. H. Smith, and B. A. Roe, 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. 143:161-178). Fragments were cloned into M13mp vectors (Messing, J., 1983. New M13 vectors for cloning. Methods Enzymol. 101:20-78) or pEMBL (Dente, L., G. Cesareni, and R. Cortes, 1983. pEMBL: A new family of single-stranded plasmids. Nucleic Acids Res. 11:1645-1654) and sequenced directly or following the generation of a set of overlapping deletions using exonuclease III (Henikoff, S., 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351-359). Sequencing was performed on both strands of the λDm4925 cDNA, the B-specific region of λDm4745 cDNA, the A- and B-specific 5' genomic regions not represented in the cDNAs, and the 3'-flanking region. The remaining exon boundaries of λDm4745 and genomic regions represented within the cDNA clones were sequenced on one strand.

D.   The E75 Gene Encodes Two Members of the Steroid Receptor Superfamily

The coding and noncoding sequences of the E75 A and B mRNAs, their splice junctions, and the 5' and 3' flanking sequences are shown in Table 1. Certain sequences of potential interest within the 5' flanking DNA and in the 5' leader mRNA sequences are indicated in the legend to Table 1. We focus here on the large open reading frames of the E75 A and B mRNAs that begin at 380 bp and 284 bp downstream from their respective mRNA start sites, each continuing into the common final exon. The termination codon in exon 5 lies upstream of both alternative polyadenylation sites; thus, the sequence of

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the encoded protein is not affected by which site is selected. Since the open reading frames in the E75 A and B mRNAs begin in the A0 and B1 exons and merge at the beginning of exon 2, the proteins encoded by the two transcription units differ in the amino-terminal region and are the same in the carboxy-terminal region. The specific amino-terminal regions contain 266 and 423 amino acid residues in the E75 A and B proteins, respectively, while their common carboxy-terminal region consists of 971 residues. The predicted molecular weights of the A and B proteins are thus 132,000 and 151,000. The open reading frames display characteristic D. melanogaster codon usage, and their extents have been confirmed by in vitro translation of mRNAs transcribed in vitro from cDNA constructs and by expression of fusion proteins in E. coli. The predicted protein sequence for each protein is punctuated by homopolymeric tracts of amino acids, which are noted in Table 1 and its legend.

Analysis of the sequences of E75 proteins and comparison to the sequences of known proteins have revealed similarity between the E75 proteins and members of the steroid receptor superfamily (Evans, R. M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240:889-895; Green, S., and P. Chambon, 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends in Genetics 4:309-314). We have used the nomenclature of Krust el al. (Krust, A., S. Green, P. Argos, V. Kumar, P. Walter, J. Bornert, and P. Chambon, 1986. The chicken oestrogen receptor sequence: Homology with v-erbA and the human oestrogen and glucocorticoid receptors. EMBO J. 5:891-897) in dividing the proteins into six regions, A to F, in the amino- to carboxy-terminal direction.

Similarity between E75A and other members of this superfamily is strongest in the C region, a cysteine-lysine-arginine-rich region that is necessary and sufficient for the binding of these receptors to DNA (for

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review, see, Evans, R. M., 1988. The steroid and thyroid
hormone receptor superfamily. Science 240:889-895;
Green, S., and P. Chambon, 1988. Nuclear receptors
enhance our understanding of transcription regulation.

5   Trends in Genetics 4:309-314). The C region consists of
66-68 amino acids, of which 20 residues are invariant
within this family. Among these are nine invariant
cysteine residues, eight are believed to coordinate zinc
in the formation of two zinc finger-like structures

10   (Miller, J., A. D. McLachlan, and A. Klug, 1985.
Representative zinc-binding domains in the protein
transcription factor IIIA from Xenopus oocytes. EMBO J.
4:1609-1614; Freedman, L. P., B. F. Luisi, Z. R. Korszun,
R. Basavappa, P. B. Sigler, and K. R. Yamamoto, 1988.

15   The function and structure of the metal coordination
sites within the glucocorticoid receptor DNA binding
domain. Nature 334:543-546; Severne, Y., S. Wieland,
W. Schaffner, and S. Rusconi, 1988. Metal binding finger
structure of the glucocorticoid receptor defined by site-

20   directed mutagenesis. EMBO J. 9:2503-2508). Within the
C region, E75A contains all of the highly conserved
residues and is approximately as closely related to other
members of the steroid receptor superfamily as they are
to one another. The closest relative of E75 appears to

25   be the human ear-1 gene, which has nearly 80% amino acid
identity to E75 A in the DNA-binding domain.

The other region conserved among members of the
steroid receptor superfamily is the E region, which is
required for steroid binding and for the linkage of

30   steroid-binding and trans-activation functions (for
review, see, Evans, R. M., 1988. The steroid and thyroid
hormone receptor superfamily. Science 240:889-895;
Green, S., and P. Chambon, 1988. Nuclear receptors
enhance our understanding of transcription regulation.

35   Trends in Genetics 4:309-314). Although overall E-region
similarity is clearly significant for the comparison of
E75 A to the thyroid hormone, vitamin D, and retinoic

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acid receptors, and ear-1, similarity to the
glucocorticoid and estrogen receptors is considerably
lower. However, the plots of local similarities show a
clear similarity to each of these proteins within three
subregions of the E region, denoted E1, E2 and E3. The
E1 subregion is the most highly conserved and corresponds
to a region shown by in vitro mutagenesis to be essential
for steroid binding and steroid-dependent trans-
activation (Giguere, V., S. M. Hollenberg,
M. G. Rosenfield, and R. M. Evans, 1986. Functional
domains of the human glucocorticoid receptor. Cell
46:645-652; Danielson, M., J. P. Northrop, J. Jonklaas,
and G. M. Ringold, 1987. Domains of the glucocorticoid
receptor involved in specific and nonspecific
deoxyribonucleic acid binding, hormone activation and
transcriptional enhancement. Mol. Endocrinol. 1:816-
822). Region E2 is less highly conserved in primary
amino acid sequence, but can, in part, be seen as a
conserved hydrophobic region in the hydropathy plots of
several of these proteins. A deletion of 14 amino acids
within this region abolished steroid binding
(Rusconi, S., and K. R. Yamamoto, 1987. Functional
dissection of the hormone and DNA binding activities of
the glucocorticoid receptor. EMBO J. 6:1309-1315). E3
falls close to the end of the region that is absolutely
required for steroid binding.

While the characteristic structural features of the
steroid receptor superfamily are well conserved in E75,
two novel variations are seen. The first of these
concerns the structure of the E75 B protein, which
contains a major alteration within its putative DNA-
binding domain. The steroid receptor superfamily DNA-
binding domain consists of two DNA-binding zinc fingers
separated by a less conserved linker region. In E75, as
in nearly all other genes of this family, an intron is
found between the two fingers. In E75, this splice marks
the beginning of the region held in common between the

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E75 A and B proteins. This results in the E75 A protein
having two fingers, while the E75 B protein has unrelated
B-specific sequences in place of the first finger. Other
sequences within the B-specific amino-proximal region may
contribute to the DNA-binding domain of the E74B protein.

Alternatively, the B protein might bind DNA with
only one finger, as GAL4 transcription factor of yeast
appears to do. It is possible that these structural
differences imply a functional difference in the DNA-
binding properties of the E75 A and B proteins that might
allow them to differentially regulate the transcription
of the late genes that characterize the secondary
response to ecdysone in different target tissues.

In this respect, it should be emphasized that the
putative hormone- or ligand-binding domain is represented
by the E region that is common to the E75A and E75B
proteins. Thus, these proteins appear to be receptors
for the same hormone, which may regulate the
transcription of different sets of genes. These proteins
represent "orphan" receptors, in that their hormone, or
binding ligand, has not yet been identified. Because
ecdysteroids are the only known steroid hormones in
Drosophila, the most obvious candidate for an E75 ligand
would be ecdysone itself. However, it is unlikely that
this is the case, since the putative hormone-binding
domain of the E75 proteins does not exhibit the high
sequence homology to that of the known Drosophila
ecdysone receptor encoded by the EcR gene (see
Experimental Example III and Table 2) that would be
expected if the E75 proteins were also ecdysone
receptors. It, therefore, seems likely that the E75
proteins would bind either a terpenoid juvenile hormone
or a novel Drosophila hormone.

The second unusual feature of the E75 proteins is
the presence of a large F region, encompassing nearly one
half of the proteins. Many of the other receptors have

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very small F regions, and no function has yet been ascribed to this region.

Methods
Protein sequence analysis

Sequence data were compiled using the Bionet system. Protein sequence comparison was performed using FASTP (Lipman, D. J., and W. R. Pearson, 1985. Rapid and sensitive protein similarity searches. Science 227:1435-1441) and Bionet IFIND programs.

E. Expression Vectors for E75 Proteins

In order to express the E75 proteins, portions of cDNAs and genomic clones were fused in order to generate cassettes containing the entire E75 A and E75 B protein coding regions. First, BamHI sites were introduced into genomic clones upstream of the initial AUGs of the large open reading frames. Then, E75 A0 exon sequences were fused to sequences of a nearly full-length E75 A cDNA, and E75 B1 exon sequences were fused to sequences of a nearly full-length E75 B cDNA. These cassettes were cloned into pGEM3 (Promega), and transcripts of the open reading frames were prepared using T7 polymerase. These were then translated in the presence of ^{35}S-methionine, and shown to give rise to proteins of appropriate size.

These cassettes have been placed into a variety of expression vectors, including pUCHsneo/Act for expression in Drosophila cells, pSV2 for expression in mammalian cells, and pOTS for expression in bacterial cells.

Methods

BamHI sites were introduced directly upstream of the initial ATGs of the E75A and E75B coding sequence -- at the SspI site upstream of the E75A initial ATG, and at the SacII site upstream of the E75B initial ATG. cDNA and genomic sequences were joined at the EcoRV site in

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the A0 exon to construct an E75A cassette, and at the
MluI in exon 3 to construct an E75B cassette.

EXAMPLE II

CLONING, STRUCTURE AND EXPRESSION OF THE EcR AND
DHR3 GENES THAT ENCODE ADDITIONAL MEMBERS OF THE
STEROID RECEPTOR SUPERFAMILY.

The following experiments were carried out after the
primary structure of the E75 gene, and of the two members
of the steroid receptor superfamily that it encodes, was
determined (Experimental, Example I). The purpose of
these experiments was to clone and determine the primary
structure of other steroid receptor superfamily genes
from Drosophila, and of the proteins they encode. The
aim was to identify the gene that encodes a Drosophila
ecdysone receptor, given that the characteristics of the
E75 gene indicated that it did not encode an ecdysone
receptor. The first stage of the experimental plan was
to use the conserved sequences in the E75A transcription
unit that encode the putative DNA-binding domain of the
E75A receptor protein as a probe to screen a Drosophila
genomic library to identify sequences encoding the
putative DNA-binding domains of other Drosophila members
of the steroid receptor superfamily. The second stage
was to isolate cDNA clones corresponding to the
identified genes, as well as additional genomic DNA
clones, to obtain the nucleotide sequence of the complete
coding region (i.e., the open reading frame encoding the
respective receptors) and the exon-intron organization of
these genes.

The experiments described below resulted in the
cloning and structural characterization of two genes that
satisfy the criteria for bona fide members of the steroid
receptor superfamily: encoding proteins that exhibit
amino acid sequence homology to both the DNA-binding and
the hormone-binding domains that are conserved among
members of this superfamily. The two genes are called

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EcR and DHR3. The EcR gene was originally called DHR23, but was renamed EcR after it was shown to encode an ecdysone receptor (see Experimental Example III). The DHR3 designation stands for Drosophila Hormone Receptor 3.

A.   Identification and Chromosomal Mapping of EcR and DHR3 Genomic Clones

Initially, Southern blots of total Drosophila genomic DNA, digested with one or more restriction endonucleases, were probed with a 530 bp fragment of the E75A cDNA containing the sequences encoding the putative DNA-binding domain of the E75A receptor protein (see Experimental Example I) at low and high stringency hybridization conditions.

To isolate the sequences responsible for these low stringency bands, this E75A probe was used to screen a Drosophila genomic library under the same low stringency conditions, counterscreening duplicate filters with E75 intron probes to eliminate phage containing inserts from the E75 gene. Five genome equivalents were screened and 39 non-E75 containing phage were isolated. The 25 most strongly hybridizing clones were divided into six classes on the basis of restriction patterns and cross hybridization, each class containing between one and six independent overlapping genomic inserts.

For each class, a restriction fragment containing the region of hybridization to the E75A probe was localized by Southern blotting. Hybridization of probes derived from these fragments to genomic Southern blots showed that each of the low stringency bands detectable by the E75A probe could be accounted for by one of the six isolated fragments.

The nucleotide sequences of the six restriction fragments were determined to test whether they represent candidate receptor genes. In all cases, DNA sequence similarities with the E75A probe were observed that are sufficient to account for the hybridization of these

fragments with the probe. When the DNA sequences were
conceptually translated in all six reading frames, four
of the fragments yielded no significant sequence
similarity with E75A at the protein level. The remaining

5 two clones, however, showed predicted amino acid
sequences with strong similarity to the DNA binding
domains of the E75A protein and other steroid superfamily
receptors.

These two clones represent the EcR and DHR3 genes,

10 as will become apparent. Probes from these clones were
used to map the position of these genes in the polytene
chromosomes by in situ hybridization. The EcR and DHR3
chromosomal loci were mapped to positions 42A and 46F,
respectively, in the right arm of the second chromosome.

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B.   Structure of the EcR and DHR3 Genes and Their
      cDNAs

The DHR3 and EcR genomic clones described above were

20 used to screen a cDNA library prepared from third instar
tissues treated with ecdysone and cycloheximide. This
procedure allowed the isolation of a large number of cDNA
clones, since both genes have a peak period of
transcription in late third instar after the rise in

25 ecdysone titer. For each gene, 20 cloned cDNAs were
purified and their lengths determined. Restriction maps
for the 10 longest cDNAs from each gene were determined
and found to be colinear.

For EcR, a 5534 bp cDNA sequence was obtained from

30 two overlapping cDNA clones. It contains an 878 codon
open reading frame (ORF) which yields a predicted amino
acid sequence expected for a member of the steroid
receptor superfamily (Table 2), as described in more
detail below. The length of the largest DHR3 cDNA that

35 was isolated (clone DHR3-9) is 4.2 kb. The nucleotide
sequence of this cDNA was determined and found to contain
a 487 codon AUG-initiated open reading frame (Table 3).
As described below, the amino acid sequence of the DHR3

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protein predicted from this sequence demonstrates that
this protein is also a <u>bona fide</u> member of the steroid
receptor superfamily.

Table 2. The cDNA sequence of the EcR gene.
Numerals at the left refer to the nucleotide sequences;
those on the right to the amino acid sequence in the EcR
5   protein. Nucleotides 1-5194 are the sequence of EcR-17
cDNA, while nucleotides 5195-5534 derive from the EcR-9
cDNA. The underlined sequences in the 5' and 3'
untranslated regions refer, respectively, to the ATG
codons and the AATAAA consensus polyadenylation signals.
10   Positions of the introns and the donor and acceptor
splice sequences are indicated above the cDNA sequence in
small type. The amino acid sequences homologous to the
conceived DNA-binding (C region) and hormone-binding (E
region) domains of the steroid receptor superfamily are
15   underlined.
Panel 1 is shown in detail in panels 2 and 3.

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Table 3.   The cDNA sequence of the <u>DHR3</u> gene.
The numbering and underlining of the nucleotide and amino
acid sequences have the same meaning as in Table 2, and
the intron positions and donor and acceptor splice
5   sequences are similarly indicated.   The sequence of the
5' proximal 2338 nucleotides of the DHR3-9 cDNA is shown.
The sequence of the remainder of this 4.2 kb cDNA was
determined for only one strand and is not shown.   Four
silent, third-position differences between the cDNA and
10   genomic DNA sequences are indicated above the cDNA
sequence.

The genomic structure of the EcR and DHR3 genes was investigated by isolating additional genomic DNA clones that form overlapping sets that contain all of the sequences found in the respective cDNA clones. The exons contained in these cDNAs were mapped within the genomic DNA by comparison of cDNA and genomic clones via Southern blot analysis, mapping of restriction cleavage sites, and finally, by determination of the nucleotide sequence of the genomic DNA in regions that contain the exon/intron boundaries. Table 2 and 3 show these boundaries and the sequence of the splice junctions for the EcR and DHR3 genes, respectively. All of these splice junctions conform to the splice donor and acceptor consensus sequences.

For EcR, the cDNA sequence shown in Table 2 is split into six exons spread over 36 kb of genomic DNA, with the ORF beginning in the second exon and ending in the sixth. For DHR3, the cDNA sequence derives from nine exons spread over 18 kb, with the ORF beginning in the first exon and ending in the ninth. Because the 5' and 3' ends of the respective mRNAs were not mapped, it should be emphasized that these genes may have additional noncoding exons at their 5' or 3' ends.

The EcR and DHR3 gene structures differ significantly from those of all previously examined steroid receptor superfamily genes. Comparison with the genes for 11 other receptor homologues for which at least partial structural information is available reveals that the positions of certain exon boundaries have been conserved in evolution. This conservation is most striking in the portion of the genes encoding DNA-binding domains. In the nine other cases where the structure of this region has been examined, the two halves of the DNA-binding domain are always encoded by separate exons. If we exclude the Drosophila genes knirps, knirps-related, and egon (which are not bona fide receptor homologues since they lack the hormone-binding domain sequence

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similarity), these are always small exons, the second one
invariably ending in the fourth codon beyond the
conserved Met codon at the end of the C region. Thus,
these exons each encode one of the two predicted zinc
fingers of the DNA-binding domain. In contrast, both
zinc fingers of the putative DNA-binding domain of the
EcR and DHR3 receptors are encoded by a single exon. It
is possible that our screen specifically selected for
genes lacking the above intron. The screen selected
genomic clones that hybridize to an E75A cDNA probe that,
of course, lacks this intron. Genomic sequences
containing a contiguous sequence encoding the DNA-binding
domain would be expected to hybridize to this probe
better than clones from genes containing the intron.
This would explain the successful isolation of the EcR
and DHR3 genes, and the failure to isolate the genes of
other Drosophila members of the steroid receptor
superfamily.

Methods
Isolation of cDNA and additional genomic clones

        Subclones of the originally isolated DHR3 and EcR
genomic clones were used to screen a cDNA library
prepared from third instar tissues treated with ecdysone
and cycloheximide. This library was chosen because both
genes are relatively highly expressed at the end of third
instar, and because of the high quality of the library.
Of the 270,000 primary plaques screened, 20 positives for
DHR3 and 220 for EcR were detected. Twenty cDNAs for
each gene were purified, of which the ten largest for
each were restriction mapped and found to be colinear.
cDNA DHR3-9, which extends further in both the 5' and 3'
directions than our other DHR3 cDNAs, was chosen for
sequencing. For EcR, the longest cDNA, EcR-17, extended
the farthest 5' and was sequenced in its entirety. An
additional cDNA clone, EcR-9, was found to extend 300 bp
farther 3' than EcR-17, and this 3' extension was also

sequenced.   Additional genomic DNA clones covering the
EcR and DHR3 genes were obtained by screening the
Drosophila Canton S genomic library referred to in part A
above, either with probes from the respective cDNA
clones, or, for overlapping clones, by the chromosomal
walk method described in Experimental Example I.

DNA sequence analysis

cDNAs were subcloned into BlueScript vectors
(Stratagene), and clones for sequencing were generated by
exonuclease III digestion (Henikoff, S., 1984.
Unidirectional digestion with exonuclease III creates
targeted breakpoints for DNA sequencing.   Gene
28:351-359).

Double-stranded plasmids were denatured (Gatermann,
K. B., G. H. Rosenberg, and N. F. Kaufer, 1988.   Double-
stranded sequencing, using mini-prep plasmids, in 11
hours.   BioTechniques 6:951-952) and sequenced by the
dideoxy chain terminating method (Sanger, F., S. Nicklen,
and A. R. Coulson, 1977.   DNA sequencing with chain-
terminating inhibitors.   Proc. Natl. Acad. Sci. USA
74:5463-5467), using the enzyme Sequenase (U.S.
Biochemical).   cDNA EcR-17 was completely sequenced on
both strands, as was the EcR-9 3' extension.   cDNA DHR3-
9 was sequenced on both strands for the 5' most 2338 bp,
which contains the entire ORF, and the remainder of the
long 3' untranslated region was sequenced on one strand.

The exon/intron boundaries in genomic DNA clones
were first mapped at low resolution by Southern blot
analysis of their restriction fragments probed with
labeled cDNAs.   Genomic DNA surrounding each exon/intron
boundary was subcloned and the nucleotide sequence of
these subclones determined as above.

Genomic exons were either sequenced entirely, or for
the longer exons, were digested and electrophoresed in
parallel with cDNA clones to confirm the colinearity of
the genomic and cDNA clones.   Shorter exons were

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completely sequenced from genomic clones. Longer exons had their boundaries sequenced from genomic clones, and were confirmed to be colinear with the cDNA clones by parallel digestion and electrophoresis of the cDNA and

5   genomic clones.

C.   The Predicted Amino Acid Sequence of the EcR
      and DHR3 Proteins and their Implications

10   Comparison of the predicted EcR and DHR3 protein sequences to the sequence database and to individual members of the steroid receptor superfamily shows that these proteins share the two conserved domains characteristic of this superfamily (Evans, R. M., 1988.

15   The steroid and thyroid hormone receptor superfamily. Science 240:889-895; Green, S., and P. Chambon, 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends in Genetics 4:309-314). We refer to the domains as the C and E regions, for the

20   more amino-terminal and more carboxy-terminal homologies, respectively, according to the nomenclature of Krust et al. (Krust, A., S. Green, P. Argos, V. Kumar, P. Walter, J. M. Bornert, and P. Chambon, 1986. The chicken oestrogen receptor sequence; homology with v-erbA and the

25   human oestrogen and glucocorticoid receptors. EMBO J. 5:891-897). These domains are underlined in Tables 2 and 3, and Table 4A-C presents a comparison of these domains from EcR and DHR3 with those from representative members of the superfamily.

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Table 4. Sequence comparison of the conserved
C and E regions in DHR3, EcR, and some representative
nuclear receptor homologues. (A) C-region alignment.
Numbers at the left indicate the amino acid positions
within the individual receptors; dashes indicate gaps
introduced to obtain maximal alignment. Dots indicate
three positions important in determining the DNA binding
specificity of this domain. (B) E-region alignment.
Bars indicate the three most highly conserved stretches
within this domain. (C) Computed percent identities
among the C-region sequences (lower left) and among the
E-region sequences (upper right). The kni sequence shows
no significant E-region homology and is, therefore, not
included in this comparison. Sequences shown are from:
E75A, Drosophila ecdysone-inducible gene at 75B; kni,
Drosophila segmentation gene knirps; hRARα, human
retinoic acid receptor alpha; htRβ, human thyroid
receptor beta; hVDR, human vitamin D receptor; cOUP-TF,
chicken ovalbumin upstream promoter transcription factor;
hERR1 and hERR2, human estrogen-related receptors 1 and
2; hER, human estrogen receptor; hGR, human
glucocorticoid receptor; hMR, human mineralocorticoid
receptor; hGR, human progesterone receptor.
    Panel 1 is shown in detail in panels 2-6.

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The C region is a 66-68 amino acid domain that
has been shown to function as a zinc finger DNA binding
domain in vertebrate receptors. This domain has also
been implicated in receptor dimerization (Kumar, V., and
5   P. Chambon, 1988. The estrogen receptor binds tightly to
its responsive element as a ligand-induced homodimer.
Cell 55:145-156). As shown in Table 4A, all 19 C-region
residues that are absolutely conserved in the other
receptor homologues are also conserved in DHR3 and EcR,
10   including the nine invariant Cys residues, eight of which
coordinate two zinc ions (Freedman, L. P., B. F. Luisi,
Z. R. Korszun, R. Basavappa, P. B. Sigler, and
K. R. Yamamoto, 1988. The function and structure of the
metal coordination sites within the glucocorticoid
15   receptor DNA binding domain. Nature 334:543-546). As
seen in Table 4C, the Drosophila C-region sequences
(including those of E75A) are not more closely related to
each other than they are to those from the vertebrate
receptor homologues. The C region of DHR3 is most
20   similar to that of the human retinoic acid receptor α
(hRARα), and the C region of EcR is most similar to that
of the human thyroid receptor ß (hTRß). Studies on the
human glucocorticoid receptor (hGR) and human estrogen
receptor (hER) have identified three C-region residues
25   (indicated by dots in Table 4A) that are critical for
determining the differential DNA binding specificity of
these receptors (Mader, S., V. Kumar, H. de Verneuil, and
P. Chambon, 1989. Three amino acids of the estrogen
receptor are essential to its ability to distinguish an
30   estrogen from a glucocorticoid-responsive element.
Nature 338:271-274; Umesono, K., and R. M. Evans, 1989.
Determinants of target gene specificity for
steroid/thyroid hormone receptors. Cell 57:1139-46).
The three Drosophila proteins DHR3, EcR, and E75A, as
35   well as the vertebrate receptors hRARα, hTRß, and the
human vitamin D receptor (hVDR), all have identical amino
acids at these three positions; thus, these proteins may

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all have similar DNA binding specificities, as has
already been shown for hRARα and hTRβ (Umesono, K.,
V. Giguere, C. K. Glass, M. G. Rosenfeld, and
R. M. Evans, 1988. Retinoic acid and thyroid hormone
induce gene expression through a common responsive
element. Nature 336:262-265).

The E-region is an -225 amino acid domain that
functions as a hormone-binding domain in vertebrate
receptors. This domain has also been implicated in
hormone dependent receptor dimerization (Kumar, V. and
P. Chambon, 1988. The estrogen receptor binds tightly to
its responsive element as a ligand-induced homodimer.
Cell 55:145-156; Guiochon, M. A., H. Loosfelt, P. Lescop,
S. Sar, M. Atger, A. M. Perrot, and E. Milgrom, 1989.
Mechanisms of nuclear localization of the progesterone
receptor: evidence for interaction between monomers.
Cell 57:1147-1154), hormone dependent nuclear
localization of the glucocorticoid receptor (Picard, D.,
and K. R. Yamamoto, 1987. Two signals mediate hormone-
dependent nuclear localization of the glucocorticoid
receptor. EMBO J. 6:3333-3340), and binding of the
glucocorticoid receptor to the 90 kDa heat shock protein
(Pratt, W. B., D. J. Jolly, D. V. Pratt,
W. M. Hollenberg, V. Giguere, F. M. Cadepond,
G. G. Schweizer, M. G. Catelli, R. M. Evans, and
E. E. Baulieu, 1988. A region in the steroid binding
domain determines formation of the non-DNA-binding, 9 S
glucocorticoid receptor complex. J. Biol. Chem. 263:267-
273). Table 4B shows an alignment of the E regions of
the DHR3 and EcR proteins with those of other receptor
homologues. The three relatively highly conserved
stretches within this region noted in Experimental
Example I are overlined; each contains a cluster of
residues conserved in all or most of the receptor
sequences. DHR3 and EcR show strong similarity to each
other and to the other proteins in these stretches, and a
lower similarity outside of them. The presence of this

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E-region homology establishes these proteins as _bona fide_
members of the nuclear receptor family, in contrast to
the _Drosophila knirps_ (Nauber, U., M. J. Pankratz,
A. Kienlin, E. Seifert, U. Klemm, and H. Jackle, 1988.
Abdominal segmentation of the _Drosophila_ embryo requires
a hormone receptor-like protein encoded by the gap gene
knirps.   _Nature_ 336:489-492), _knirps-related_ (Oro, A. E.,
E. S. Ong, J. S. Margolis, J. W. Posakony, M. McKeown,
and R. M. Evans, 1988.   The _Drosophila_ gene _knirps-related_ is a member of the steroid-receptor gene
superfamily.   _Nature_ 336:493-496), and _egon_ (Rothe, M.,
U. Nauber, and H. Jackle, 1989.   Three hormone receptor-like _Drosophila_ genes encode an identical DNA-binding
finger.   _EMBO J._ 8:3087-3094) proteins, which show C-region homology but no E-region homology.   The E region
in DHR3 is most similar to that of E75A, and the E region
of EcR is most similar to that of hTRß, although the
level of these similarities is lower than those found
among E regions of many other receptors (Table 4C).
Thus, DHR3 and EcR are not especially close homologues of
any previously cloned receptors.   Comparison of E-region
sequences allows division of the nuclear receptors into
subfamilies (Petkovich, M., N. J. Brand, A. Krust, and
P. Chambon, 1987.   A human retinoic acid receptor which
belongs to the family of nuclear receptors.   _Nature_
330:444-450), the members of any one subfamily being more
related to each other than to those in other subfamilies.
The DHR3 and EcR receptors fall into a subfamily with the
E75A, E75B, hRARα, hTRß, and hVDR receptors.

D.   In Situ Labeling of the EcR and DHR3 Proteins
     with Antibodies Induced by Proteins Produced in
     E. coli

To determine the intracellular and tissue
distribution of the EcR and DHR3 proteins in _Drosophila_,
affinity-purified polyclonal antibodies directed against
those proteins were produced in the following manner.
The region of about 120 amino acid residues located

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between the conserved DNA-binding and hormone-binding domains of these proteins was used as the immunogen to produce antibodies against each protein. Thus, the coding sequences for amino acids 335-447 of the EcR protein and for amino acids 164-289 of the DHR3 protein (see Tables 2 and 3, respectively) were cloned into the appropriate pATH (Dieckmann, C., and A. Tzagaloff, 1985. J. Biol. Chem. 260:1513-1520) or pUR expression vectors, so as to fuse these coding sequences to those encoding E. coli β-galactosidase (βgal) or to E. coli tryptophan E protein (trpE), respectively.

The βgal fusion proteins were produced in E. coli by the addition of the IPTG inducer to exponential cultures, while the production of trpE fusion proteins were induced by dilution into tryptophan-free media and subsequent addition of indoleacetic acid. For EcR, the trpE fusion protein was used as an immunogen and the βgal fusion protein was used on immunoblots to test sera for immunoreactivity to the EcR portion of the fusions. For DHR3, the βgal fusion protein was injected, and sera were checked against the trpE fusion protein.

For immunization the appropriate fusion protein was prepared by electrophoresis on SDS-PAGE gels and visualized by staining in ice-cold 0.25 M KCl, after which the fusion protein band was cut out. Approximately 100 µg of fusion protein in 0.25 ml of gel slice was crushed by passing through successively smaller hypodermic needles, and mixed with 0.25 ml of a sterile saline solution and 0.5 ml of Freund's complete adjuvant. For each immunogen, two New Zealand White rabbits were injected at multiple intramuscular sites, and after one month, boosted at two-week intervals, omitting the Freund's adjuvant. While the βgal fusion proteins were subject to the above gel electrophoresis without prior purification, the trpE fusion proteins were first purified by the following method which takes advantage of their insolubility in vivo.

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E. coli from a two-liter culture of induced cells were washed, and the cell pellet was subjected to several freeze/thaw cycles. The cells were resuspended in 18 ml of 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 1.8 ml of 10 mg/ml lysozyme was added. After 15 minutes on ice, the cells were lysed by passing three times through a french pressure cell at 10,000 psi. The insoluble fraction was collected by centrifugation at 27,000 x g for 15 minutes, and washed by resuspension, using a Dounce homogenizer, in ice-cold 50 mM Tris-HCl, 0.5 mM EDTA, 0.3 M NaCl, followed by centrifugation as above. The washing step was repeated, and the final pellet dissolved in 10 ml of 4M urea, 2% (w/v) SDS, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5% (v/v) 2-mercaptoethanol. Material remaining insoluble was centrifuged out and discarded.

The antisera was affinity purified in a two-step procedure by successive passage through "nonspecific" and "specific" affinity columns. In the case of antibodies raised against the trpE fusion proteins, the nonspecific column consisted of resin coupled to the insoluble protein derived from E. coli expressing unmodified trpE protein, and was used to remove antibodies directed against trpE epitopes, as well as against insoluble E. coli protein impurities. The specific column consisted of resin coupled to the EcR-trpE fusion protein (purified as described above) and was used to absorb the desired antibodies directed against the EcR epitopes, antibodies that were subsequently released from the column. In the case of antibodies raised against the ßgal fusion proteins, the same general procedure was used, except that the resin in the nonspecific column was coupled to ß-galactosidase, while that in the specific column was coupled to the DHR3-ßgal fusion protein. Western blot analysis of the appropriate E. coli extracts demonstrated that these affinity-purified antibodies exhibited the desired specificity.

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The intracellular distribution of the EcR protein in late third instar salivary glands was examined by in situ labeling of this protein with the anti-EcR antibody. The EcR protein was thereby shown to be highly localized in the nuclei of these glands. Indeed, when the polytene chromosomes in these nuclei were examined by the antibody-labeling method of Zink and Paro (Zinc, B., and R. Paro, 1989. Nature 337:468-471), specific loci within these chromosomes exhibited strong binding of the EcR protein. In particular, the EcR protein was bound to the early puff loci, including those occupied by the E75 and E74 genes. This is the result expected if the ecdysone receptor encoded by the EcR gene is that which induces the transcription of the early genes, as anticipated by the Ashburner model. Another prediction of the Ashburner model is that the ecdysone-receptor complex initially represses the genes responsible for the later puff, so that the transcription of the late genes induced by the early gene proteins is delayed until these proteins accumulate sufficiently to overcome this initial repression. If the EcR receptor is involved in this postulated initial repression, then one would expect the EcR protein to bind to the late puff loci in the salivary glands. This expectation was met by the observation that EcR protein also binds to the late puff loci in the polytene chromosomes.

Additional in situ antibody labeling experiments demonstrated that the EcR protein is present in the nuclei of all ecdysone target tissues examined in late third instar larvae. It is also present in most, if not all, cells during embryogenesis and other stages of Drosophila development that have been examined. In this respect, the EcR protein was not detected by anti-EcR antibody labeling of embryos in which the EcR gene was eliminated by a chromosomal deletion, further demonstrating the specificity of this antibody.

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In contrast to the widespread distribution of the EcR protein, anti-DHR3 antibody labeling of embryos demonstrated that the distribution of the DHR3 protein is highly restricted during this stage of development. During the brief embryonic period of expression, the protein is restricted to the peripheral nervous system, and to cells surrounding the spiracles at the posterior end of the embryo.

Finally, it should be noted that affinity-purified antibodies against the E75A protein have also been prepared by the same technique described above for anti-EcR and anti-DHR3 antibodies. In situ antibody labeling of the E75A protein in larval salivary glands has also demonstrated that this protein is localized in the nucleus and is bound to specific loci in the polytene chromosomes.

EXAMPLE III

THE ECDYSTEROID-BINDING, DNA-BINDING AND GENETIC REGULATORY PROPERTIES OF THE EcR PROTEIN DEMONSTRATE THAT IT IS AN ECDYSONE RECEPTOR.

The following experiments demonstrate that the protein encoded by the EcR gene is an ecdysone receptor by the following three criteria. (1) The EcR protein binds ecdysteroids and accounts for a large proportion, if not all, of the ecdysteroid-binding activity present in Drosophila embryos and in a variety of cultured Drosophila cells. (2) The EcR protein binds with high specificity to a DNA sequence that functions as an ecdysone response element (EcRE), i.e., an enhancer that confers ecdysone inducibility to a promoter. (3) Cells that do not respond to ecdysone because they lack functional ecdysone receptors are transformed to the ecdysone-responsive state by transfection with an EcR expression plasmid.

A.   The EcR Protein Binds Ecdysteroids

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The EcR expression plasmid, pMTEcR, shown in
Figure 1 contains the open reading frame encoding the EcR
protein (EcR ORF; see Experimental Example II) fused to
the Drosophila metallothionine promoter (P_{MT}) at its 5'

5   end, and the polyadenylation-cleavage sequences of the
Drosophila Actin 5C gene at its 3' end. Because
transcription of the EcR ORF is under the control of this
metallothionine, that transcription is induced by Cu^{2+} ion
to yield an mRNA that, in turn, produces the EcR protein.

10   A cell line, MtEcRHy, that overproduces this protein upon
Cu^{2+} induction, as determined by Western blot analysis
using the affinity-purified anti-EcR antibody (see
Experimental Example II), was constructed by the stable
integration of the pMTEcR plasmid DNA into the genome of

15   Drosophila Sch-2 cell line. A control cell line, MtHy,
was similarly constructed by the integration of the
expression vector DNA lacking the EcR ORF.
        Whole cell extracts were prepared from both the
MtEcRHy and MtHy cell lines after Cu^{2+} induction, and were

20   assayed for ecdysteroid-binding activity using the high
affinity ecdysone analogue [^{125}I] iodoponasterone A. The
MtEcRHy extract contained sevenfold more saturable
ecdysteroid-binding activity than the MtHy control
extract.

25   To see if the induced ecdysteroid-binding activity
was due to the EcR polypeptide itself, the EcR protein
was depleted from the MtEcRHy extract by
immunoprecipitation using an affinity-purified anti-EcR
polyclonal antibody, or, as a control, the extract was

30   mock-depleted with preimmune serum. The treated extracts
were then assayed for ecdysteroid-binding activity.
Comparison of the immuno-depleted extract with the mock-
depleted extract showed that most of the binding activity
was removed by the anti-EcR antibody treatment,

35   indicating that the induced ecdysteroid-binding activity
results from the EcR protein.

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The endogenous ecdysteroid-binding activity in the control cell line, MtHy, was unchanged by Cu^{2+} exposure, and was approximately the same as that in the Sch-2 cell from which it derives. The question arises as to whether the endogenous activity in these and other Drosophila cell lines, as well as in embryonic extracts, results from the expression of the EcR gene in their respective genomes. To answer this question, extracts from embryos and several cell lines were immuno-depleted and mock-depleted, as described above, and assayed for ecdysteroid-binding activity. Again, comparison of these treated extracts showed that the large majority of the endogenous binding activity was removed in each case by treatment with the anti-EcR antibody. Thus, it appears that most, if not all, of the endogenous binding activity in embryos and cell lines results form the resident EcR gene.

Methods

Extracts

Tissue culture cell extracts for hormone and DNA-binding experiments were prepared as follows. Cells were grown in spinner flasks to a density of $5-7 \times 10^6$ cells/ml, and were washed once in EcR buffer (25 mM Hepes, pH 7.0, 40 mM KCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, and the following cocktail of protease inhibitors: 10 mM $Na_2S_2O_5$, 500 μM PMSF, 1 μM leupeptin, 1 μM pepstatin). All further manipulations were at 4°C. Cells were resuspended in EcR buffer at 2% of the original culture volume, divided into 3 ml aliquots, and sonicated using 30 1/2 second pulses with a probe sonicator (Bronson Sonifier 450), resulting in disruptions of ~95% of the cells. After centrifugation at 100,000 x g for 1 hour, 100 μl aliquots of supernatant were frozen in liquid nitrogen, and stored at -80°C. Protein concentration was determined using bovine serum albumin as the standard, and was typically 6-11 mg/ml.

Embryo extracts were prepared by a similar protocol: 3-6 hour Canton S embryos were dechorionated in 55% commercial bleach for 2 minutes, washed extensively in 0.7% NaCl, and resuspended using 2 grams of embryos per ml of EcR buffer. Embryos were broken with 20 strokes in a Dounce homogenizer using a B pestle, and lysis was completed with the probe sonicator using the same settings as used for the tissue culture cells. The extract was adjusted to 400 mM KCl, centrifuged 1 hour at 100,000 x g, and aliquots of supernatant were frozen. This extract contained 13.4 mg/ml protein. Before use in hormone binding, it was diluted tenfold in EcR buffer lacking KCl to bring the final KCl concentration to 40 mM.

Hormone-binding assays

For hormone-binding experiments, extracts were first diluted to the following concentrations in EcR buffer: 0.9 mg/ml for MtHy and MtEcRHy extracts, 3 mg/ml for S2 and SRS 1.5 extracts, 4 mg/ml for the Kc cell extracts, and 1.3 mg/ml for the embryo extract. All manipulations were done on duplicate samples in order to quantify variability in the results. For immunoprecipitation experiments, extracts were immuno-depleted, mock-depleted, or left untreated. For depletions, 300 μl of diluted extract was incubated for 30 minutes at 25°C with 3.5 μl affinity-purified anti-EcR antibody, or with 3.5 μl preimmune serum for the mock-depletion control. Then 38 μl 10% Staphylococcus aureus (Pansorbin, Calbiochem) in EcR buffer was added, and incubation was continued for 15 minutes at 25°C. After centrifugation for 3 minutes in a microcentrifuge, the supernatant (depleted extract) was recovered. The immunoprecipitation was repeated, except in the case of the embryo extract, which was subjected to only one round of precipitation. The "untreated" extract aliquots were left at 4°C for the duration of the depletion procedure,

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and were diluted with EcR buffer to match the final
concentration of the depleted aliquots.

A modification of the hormone-binding assay of P.
Cherbas was used (Cherbas, P. 1988. Proc. Nat'l Acad.
5   Sci., U.S.A. 85:2096-2100). Assay tubes contained 140 μl
extract, 14 μl [^{125}I] iodoponasterone, and either 14 μl
EcR buffer or 14 μl unlabelled 20-OH ecdysone in EcR
buffer as a competitor. [^{125}I] iodoponasterone was 2177
Ci/mM and was used at a final concentration of 5×10^{-10} M in
10   the assay; 20-OH ecdysone was 2×10^{-5} M final concentration
in the assay. After incubation for 1 hour at 25°C, each
reaction was spotted on a dry Whatman GF/C filter
(2.4 cm), and after 30 seconds the filter was washed by
using a vacuum to draw 10 ml EcR buffer through the
15   filter over a period of 1 minute. Filters were placed in
800 μl 4% SDS, and radioactivity was measured in a γ
counter. The hormone-binding activities shown are
saturable binding activities, calculated as the total
binding activity, as measured in assays with no added
20   competitor, minus the unsaturable binding activity, which
was measured in the assays with excess unlabelled
ecdysone added. In the most active extracts, the
unsaturable activity (representing the large number of
low affinity binding sites in the extract) was less than
25   10% of the total activity.

B.   Genetic Regulatory Activity of the EcR Protein
       in vivo

30   An ecdysone-inducible reporter plasmid,
pEcRE/Adh/ßgal (Figure 2), was constructed to test the
regulatory functions of the EcR protein in vivo. The
reporter gene in this plasmid consists of the sequence
that encodes the E. coli ß-galactosidase (ßgal ORF)
35   linked through the 5' leader sequence of the Drosophila
Ultrabithorax gene (UBX leader and AUG) to an ecdysone-
inducible promoter. This promoter was created by fusing
a truncated version of the proximal promoter for the

Drosophila Adh gene ($P_{DAdh-34+53}$, the numbers indicating that it consists of the sequence from base pair positions -34 to +53, which just includes the TATA box) to seven repeats of a 34 bp synthetic oligonucleotide (7 EcRE OLIGOS) which contains the ecdysone response element (EcRE) from the ecdysone-inducible heat shock gene hsp 27 (Riddihough and Pelham, 1987. EMBO J. 6:3729-3734). The seven EcREs should confer ecdysone-inducibility to the truncated promoter, provided that the cells transfected with this reporter plasmid contain the appropriate ecdysone receptor.

This ecdysone-inducible reporter plasmid was constructed by insertion of the 7 EcRE OLIGOS into plasmid pAdh/ßgal, which is identical to pEcRE/Adh/ßgal except that it lacks the array of ecdysone response elements. The pAdh/ßgal plasmid should therefore not be ecdysone inducible and can serve as a control. To test these expectations, Sch-2 cultured cells (which were shown above to contain endogenous ecdysone-binding activity) were transfected with each plasmid and examined for ß-galactosidase activity in the presence and absence of ecdysone. The ecdysone-induced ß-galactosidase activity in the pEcRE/Adh/ßgal transfected cells was 2000-fold greater than when such cells were not exposed to ecdysone, whereas ecdysone had little effect on the pAdh/ßgal transfected cells. These results indicate that the EcREs confer ecdysone-inducibility on the $P_{DAdh-34+53}$ promoter, as expected, and that the Sch-2 cells contain functional ecdysone receptors.

To test the function of the EcR receptor in such a system, host cells lacking functional ecdysone receptors are required. "Ecdysone-resistant" cells lacking ecdysone-binding activity, and hence, presumably functional receptors, can be produced by continuously exposing ecdysone-responsive cells to ecdysone during a period of several weeks. This ecdysone-resistant state is then maintained in ecdysone-free media for several

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months. An ecdysone-resistant cell line, SRS 1.5, was therefore generated by growing Sch-2 cells in 3×10^{-6} M ecdysone. The SRS 1.5 cells lack significant ecdysone-binding activity.

5   When these cells were transfected with the pEcRE/Adh/ßgal plasmid and subsequently exposed to ecdysone, very little ecdysone-induced ß-galactosidase activity was observed, indicating that the cells have only trace amounts, if any, of functional receptors. To
10   test whether the expression of the EcR gene can "rescue" this deficiency, the SRS 1.5 cells were cotransfected with two plasmids: the ecdysone-inducible reporter plasmid, PEcRE/Adh/ßgal, and a constitutive expression plasmid for the EcR gene, pActEcR, in which transcription
15   of the EcR ORF is controlled by the Drosophila Actin 5C promoter, P_{Act5C} (Figure 3). Cotransfection with these two plasmids, followed by exposure to ecdysone, resulted in a dramatic induction of ß-galactosidase activity. Thus, introduction of this EcR expression plasmid into the
20   SRS 1.5 cells regenerated the ecdysone-inducibility they had lost.

Methods

Construction of the pAdh/ßgal, pEcRE/Adh/ßgal and pActEcR
25   plasmids

        Plasmid pAdh/ßgal was constructed in two steps. The BglII-ScaI fragment of pDΔ5'-34, containing nucleotides -34 to +53 of the Drosophila Adh distal promoter, was
30   cloned into pUC18 cut with ScaI and BamHI. The resulting plasmid was cut with EcoR1, and the EcoR1 fragment of cPßbxd6.2 (containing the Ubx untranslated leader and AUG, the ßgal open reading frame, and the SV40 splice and poly A signals) inserted.
35   To construct pEcRE/Adh/ßgal from pAdh/ßgal, two 34-residue oligonucleotides were synthesized:

5'TCGAGAGACAAGGGTTCAATGCACTTGTCCAATG3'
    3'CTCTGTTCCCAAGTTACGTGAACAGGTTACAGCT5'

These will anneal to form 30 bp duplexes with SalI
compatible four nucleotide overhangs at their 5' ends, as
shown. Further annealing via the 5' overhangs allows
formation of tandem arrays that can be inserted into
pAdh/ßgal at its SalI site just upstream from the TATA
box of the truncated Adh promoter. When these
oligonucleotides were kinased, annealed, ligated into
SalI-cut pAdh/ßgal and cloned, pEcRE/Adh/ßgal was
obtained. Restriction mapping showed that it contained a
tandem array of seven 34 bp repeats, each of which
contains the 23 bp ecdysone response element (EcRE)
present in the hsp 27 gene, the remaining 11 bp
representing flanking hsp 27 sequences and the 5'
overhangs.

The constitutive EcR expression plasmid, pActEcR,
was formed by inserting the Fsp1-HpaI fragment of an EcR
cDNA containing bp 851-4123 that contains the ORF
encoding the EcR protein (Table 2), into the EcoRV site
of the ActSV40BS plasmid. This expression vector was
constructed in two steps by inserting the Xba1-EcoR1
fragment of cosPneoß-gal, containing the SV40 splice and
poly A signals, into BlueScript+KS (Stratagene) cut with
SacII and Xba1, blunting the EcoR1 and SacII ends. The
resulting plasmid was digested with BamH1 and Apa1, and
the BamH1-EcoR1 fragment of pPAc was inserted, with the
Apa1 and EcoR1 ends being blunted.

Transfection and generation of the cell line SRS 1.5

The cell line SRS 1.5 was obtained by growing
Schneider line 2 (Sch-2) cells in the presence of 3×10^{-6} M
20-OH ecdysone (Sigma). This treatment initially halts
growth of Sch-2 cells, but after several weeks the
adapted cells grow well. SRS 1.5 cells were washed in
hormone-free medium and passed several times in hormone-
free medium prior to their use in transfection
experiments. Cells were transfected by the calcium
phosphate technique. Cells were transfected with 10 µg

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of each plasmid used; when only a single plasmid was
being transfected, 10 μg of pUC18 DNA was added as a
carrier. In general, all transfections were carried out
in duplicate. Twenty-four hours after transfection,
5   cells that were to undergo hormone treatment were split
into two dishes, one of which was treated with 2×10^{-6} M
20-OH ecdysone.

β-galactosidase assays

10   Forty-eight hours after transfection, 2 ml of cells
were washed once in PBS (137 mM NaCl, 27 mM KCl, 65 mM
Na_2HPO_4, 15 mM KH_2PO_4, pH 6.8), and were resuspended in
50 μl of 0.25 M sucrose, 10 mM Tris, pH 7.4, 10 mM EDTA,
and repeatedly frozen in liquid nitrogen and thawed in a
15   37°C water bath for a total of 3 freeze/thaw cycles.
Cell debris was removed by a 10-minute centrifugation in
a microcentrifuge at 4°C. The concentration of protein
in the supernatant (cell extract) was determined by the
Bradford method, with bovine serum albumin as a standard,
20   and was typically 1.5-2.5 mg/ml. Extracts were assayed
immediately or frozen and assayed up to two weeks later
with no loss in activity. To 10 μl of extract, or an
appropriate dilution, 500 μl of assay buffer was added
(0.6 mM 4-methylumbelliferyl-β-D-galactoside, 60 mM
25   Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.0 mM $MgSO_4$, pH 7.0).
After a 30-minute incubation at 37°C, reactions were
stopped with 500 μl of 300 mM glycine, 15 mM EDTA,
pH 11.2. The fluorescent reaction product was quantified
on a Perkin-Elmer LS-5B luminescence spectrometer, with
30   λ_{ex}=365 nm and λ_{em}=450 nm. βgal activities are given as
fluorescence units per μg protein assayed.

       C.   Specific Binding of the EcR Protein to Ecdysone
             Response Elements
35

       The simplest explanation of the results described in
the preceding section is that the EcR protein generated
by the EcR expression plasmid binds to the EcRE of the

reporter plasmid and, in combination with ecdysone, activates the minimal _Adh_ promoter in that plasmid. The following experiment was designed to test whether the EcR protein exhibits specific binding to this EcRE _in vitro_.

Two plasmids were used: pUC18, which serves as the control, and pUC18-EcRE, which was generated by substituting the HindII-XbaI fragment from pEcRE/Adh/βgal that contains the seven repeats of the 34 bp EcRE oligonucleotide, for the HindII-XbaI fragment of pUC18. Because the only difference between these two fragments is the seven oligonucleotide repeats, this is also the only difference between the two plasmids.

The two plasmids were digested with ApaLI and Hind III, end-labeled with ^{32}P and mixed with an extract from MtEcRHy cells in which the EcR protein was overexpressed by Cu^{2+} induction (see section A, above). After a 15-minute incubation at 25°C to allow EcR-DNA binding to occur, affinity-purified anti-EcR antibody was added. The 25°C incubation was continued for an additional 40 minutes, at which time anti-rabbit Ig-coated magnetic beads (Dupont Magnasort-R) were added, and the incubation continued 15 minutes more. The beads were separated from the solution magnetically, similarly washed, and the DNA eluted from the beads in 1% SDS at 65°C. The eluted DNA was ethanol precipitated and fractionated by electrophoresis in an agarose gel, which was dried and autoradiographed.

Only the fragment containing the EcRE oligonucleotide was specifically and efficiently registered on the autoradiographs, and that registration was dependent upon the anti-EcR antibody. Quantitative analysis of the autoradiographs demonstrated a 10^3-fold preference for binding to the EcRE oligonucleotide over the average vector sequences, under the conditions of this assay (see Methods, below).

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According to the criteria stated at the beginning of this Experimental Example, the EcR protein clearly satisfies the definition of an ecdysone receptor.

5   Methods

Conditions for the DNA binding assay

A quantity of 0.2 fmole of digested, labelled plasmid DNA was mixed with 2 μg (dI/dC) in 10 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and 90 μl of the

10   MtEcRHy extract, diluted to 0.9 mg/ml in EcR buffer adjusted to 180 mM KCl, was added. After binding for 15 minutes at 25°C, 2 ml of affinity-purified anti-EcR antibody, diluted 1.5x in EcR, was added, and this incubation was continued at 25°C for 40 minutes, when

15   50 μl of anti-rabbit Ig-coated magnetic beads (Dupont Magnasort-R), exchanged into 180 mM KCl EcR buffer, was added and the incubation continued for 15 minutes.

The beads were washed twice in 400 μl 180 mM KCl EcR buffer, and DNA was eluted from the beads by soaking

20   twice in 200 μl 1% SDS in TE at 65°C. The eluted DNA was ethanol precipitated and run on an agarose gel, which was dried and autoradiographed. As controls, one half of the input DNA (0.1 fmole) was run on the gel for comparison, and the binding assay was carried out, leaving out the

25   antibody.

EXAMPLE IV

RECEPTOR GENE MUTAGENESIS.

30   Mutations in the steroid receptor superfamily genes can alter their function in two ways. Most obviously, they alter the sequences encoding the receptor proteins and thus alter the receptor function. Alternatively, they can alter the expression of these genes -- an

35   alteration that can be at any level of that expression from transcription of the gene to the translation of its mRNA(s). Such mutations can change the timing of gene expression during development or change the tissue and

cell distribution of that expression, thus, profoundly changing the course of development. Furthermore, these mutations provide information about the regulation of receptor gene expression, just as mutations that alter the structure of the receptors encoded by these genes provide information about the genes whose expression these receptor proteins control. In particular, mutations that alter receptor gene expression can lead to the identification of the proteins and other regulatory molecules that control their expression. Clearly, mutagenesis of insect steroid receptor superfamily genes provides an important avenue leading to an ability to interfere in a highly specific manner with insect development, and thus to control insect infestations deleterious to human health and agriculture.

We have carried out mutagenesis experiments for two Drosophila members of the steroid receptor superfamily genes, E75 and EcR, that we have cloned and characterized with respect to their expression. In this experimental example, mutagenesis of the E75 gene is described.

A.   Deletion Mutations

In Drosophila, genetic analysis for a given locus -- in this case, the early puff locus at 75B that houses the E75 gene -- generally depends upon the isolation of deletions of all or part of that locus. Such deletions greatly facilitate the subsequent isolation of point and other small mutations within the locus. By isolating mutations that are revertants to the neighboring dominant Wrinkled (W) mutations, we have isolated and molecularly mapped the boundaries within our chromosomal walk (see Experimental Example I) of two deletions, W^{R4} and W^{R10}, generated by gamma ray mutagenesis, the preferred way of generating such large alterations of genomic structure. One of these, W^{R10}, extends distally from Wrinkled to cover the entire E75 gene; and the other, W^{R4}, extends to a point about 90 kb upstream of the 5' end of the 50 kb

E75A transcription unit and does not include the E75 gene.

An F2 screen was then employed to screen for gamma ray-induced mutations mapping to the 200 kb distal region that is included in the W^{R10} deletion but not the W^{R4} deletion. This screen resulted in the isolation of five members of a single lethal complementation group that molecular mapping data demonstrate represents the E75 gene. The most useful of these five mutations is the $E75^{X48}$ mutation. Molecular mapping of this mutation demonstrated that it is a 105 kb region that includes all of the E75 gene. This method provides an extremely efficient screen for other E75 mutations, i.e., by screening for mutations that cannot complement this deletion mutation.

B. E75 Mutations Generated by Ethyl Methane Sulfonate

The chemical mutagen ethyl methane sulfonate (EMS) was used for this screen, as it is the preferred method for generating point or small mutations. An F2 screen of 15,000 lines resulted in the isolation of 23 penetrant mutations within the 105 kb region of the $E75^{X48}$ deletion, all of which turned out to be alleles of E75. It appears that this 105 kb region was saturated by this screen with respect to lethal complementation groups, and hence, E75 appears to be the only lethal complementation group in this region. Adding the five E75 mutations described above, a total of 28 penetrant E75 alleles have thus been isolated, several of which are temperature-sensitive alleles.

Inter se complementation studies among these alleles and examination of their phenotypes reveal a complex complementation group -- a complexity that probably results from the fact that the E75 gene contains two overlapping transcription units, a 50 kb E75A unit and a 20 kb E75B unit that occupies the 3' end of the E75A unit

(see Experimental Example I and Table 1). These alleles can be roughly divided into two groups: (1) those that cause lethality in early development, during the latter part of embryogenesis or during early larval development, and (2) those that cause lethality late in development, during the prepupal or pupal stages.

This division correlates with the stages when the E75A and E75B units are expressed. Thus, E75A transcription is associated with each of the six pulses of ecdysone, including those that mark the embryonic and early larval stages. By contrast, E75B mRNAs are not observed until the end of the last larval stage, being particularly abundant during the pupal stage. This correlation invites the speculation that the early lethal mutations affect the expression of the E75A unit and its E74A protein, and that the late lethal mutations specifically affect the expression of the E75B unit and its E75B protein. This proposition can be tested by detailed molecular mapping of these mutations and further examination of their phenotypes at the molecular level to determine the causes of lethality.

The mutants described here provide a foundation for the further genetic analysis of the E75 gene that will allow exploration of the requirements for appropriate E75 expression and function and will identify structural and functional domains of E75. Some of the future E75 studies will best be performed by its in vitro manipulation, followed by transformation of the constructs back into Drosophila. Finally, it will be desirable to identify interacting genetic loci -- interactions that may occur at the level of regulation of E75 expression or at the level of interaction of the E75 proteins with those encoded by other genes. Such interactive genetic loci can be identified via the isolation of mutations that act as suppressors or enhancers of the E75 mutations.

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Methods

Strains, markers and chromosomes

For this aspect of the invention, the following strains, markers and chromosomes were used. Tu^2 was described by Lindsley (Lindsley, 1973. DIS 50:21). All other strains and mutations are as described (Lindsley, and Grell, 1968. Genetic Variation of Drosophila melanogaster, Publication 627, Carnegie Institute of Washington, Washington, DC). ru h W^{R4} e^s ro ca was constructed by recombination between ru h W^{R4} sbd^2 Tu^2 and st sbd^2 e^s ro ca. The st in ri p^p sbd^2 chromosome was constructed by recombination of st in ri p^p with sbd^2, in order to allow marking of this chromosome over W^{R4} and W^{R10}, and homozygosed by crossing to TM3, backcrossing to TM3, and mating of isogeneic sibling progeny. The st p^p ell line was homozygosed by standard ionic procedures. $Antp^W$ and ns^{RC4} are described in Scott et al. (1984) Proc. Nat'l Acad. Sci. USA 81:4115-4119. The pupal lethals X19, g26, O13B, 8m12, iX-14, 2612, m45, p4, q30L, mz416, 13m115, O52 and wq49 are described in Shearn (1974) Genetics 77:115-125. All strains used to construct the strains described above and other strains were obtained from the Bowling Green and Caltech stock centers.

TM1, TM3 and TM6B (Lindsley, and Grell, 1968. Genetic Variation of Drosophila melanogaster, Publication 627, Carnegie Institute of Washington, Washington, DC) are balancer chromosomes carrying recessive lethal mutations along with multiple inversions to suppress recombination. This allows the maintenance, as a heterozygote, of a recessive lethal chromosome in its original state. These chromosomes are also marked with convenient visible markers.

Quantitative Southern blot mapping for detection of mutant lesions

DNA was prepared from adult flies (about 50) by
douncing in 1 ml of 10 mM Tris-HCl, pH 7.5, 60 mM NaCl,
10 mM EDTA, 0.15 mM spermine, 0.2 mg/ml proteinase K.
The homogenate was added to an equal volume of 0.2 M
Tris-HCl, pH 9.0, 30 mM EDTA, 2% SDS, 0.2 mg/ml
proteinase K, incubated at 37°C for 1 hour, and then
extracted twice with buffer-saturated phenol and once
with 24:1 chloroform/isoamyl alcohol. DNA was EtOH
precipitated twice, hooking the pellet out without
centrifugation. Southern blot hybridization was as
described (Segraves, W. et al., 1984. _J. Mol. Biol._
175:1-17). Where restriction fragment length
polymorphism was not used in order to distinguish the
parental chromosome from the balancer chromosome,
quantitation of band intensity on genomic Southerns was
achieved using a scanning densitometer. By using a
control probe outside the mutant region, the amount of
DNA in each track was internally controlled. Comparison
of deficiency heterozygote to wild type bands, when
normalized to a control band in this way, gives little
deviation from the expected 1:2 ratio.

Molecular cloning of mutant lesions

Restriction fragments of the appropriate size were
isolated by preparative low melting agarose (FMC)
electrophoresis of about 20 μg of restricted genomic DNA.
The 6 kb W^{R4} XhoI fragment was cloned into XhoI-cleaved
λSE6DBam which is propagated as a plasmid in order to
grow the vector and cannot be packaged without an insert.
The 18 kb W^{R10} SalI fragment was cloned into the SalI site
of λEMBL3, cleaved also with EcoRI for the biochemical
selection method for the prevention of propagation of
non-recombinant clones. The 7 kb EcoRI fragment
containing the x37 breakpoint was cloned into EcoRI-
cleaved λ607. Plating of recombinants on the hflA strain
RY1073 prevented plaque formation by non-recombinant
phage. The 14 kb x48 EcoRI fragment was cloned into the

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EcoRI site of λEMBL4, which had been cleaved with BamHI
to utilize the "biochemical selection" for recombinants.
The breakpoint fragments of x44 and the recipient
fragment were cloned into λSE6ΔBam. Libraries were
packaged using λ in vitro packaging extracts prepared as
described in Hohn (Hohn, B., 1979. Methods Enzymol.
68:299-303). After demonstration that each of the
libraries gave a significant number of plaques only when
inserts were included in the ligation, they were screened
using restriction fragments capable of detecting the
breakpoint clones.

Gamma ray mutagenesis

Adult males of the strain ru h W sbd^2 Tu^2 or st in ri
p^p sbd^2 were irradiated in plastic vials with 5000 rad of
gamma rays from a Cs^{137} source at a dose rate of 4300
rad/minute. These were then mated to virgins of the
appropriate strain, which were allowed to lay eggs for
five days.

EMS mutagenesis

The primary lesion in EMS-induced mutations of
bacteria and yeast is an alkylation-induced transition of
guanine to adenine; most EMS-induced point mutations in
Drosophila can similarly be explained on this basis.
This change would be expected to convert, on the
complementary strand, a C in the opa repeat element to a
T, creating an in-frame stop codon (CAGCAA to UAGCAA or
CAGUAA). (Ethylnitrosourea, ENU, which has been reported
to yield a higher number of mutations for a given amount
of sterility, is also an alkylator; however, considerably
more stringent precautions must be taken in handling this
mutagen.)

EMS was administered at 0.025 M to unstarved 1.5-5
day-old males in 1% sucrose solution (1.5 ml on two slips
of Whatman #2 in a 350 ml milk bottle). Starvation of
the males for 8 hours before EMS administration resulted

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in unacceptable levels of sterility, and males of the st p^p e^{11} strain readily fed upon the EMS/sucrose solution without starvation. Mutagenesis was monitored by crossing mutagenized males to attached-X FMA3 females. Other mutants seen in this screen included a large number of ca alleles (many mosaic) seen over TM6B in the F1 and F2 generations, a dominant brown allele, and two new mutants, Wink, a third chromosome dominant mutation resembling Bar, and a third chromosome dominant Curly-like mutation. Wink is easily scored (RK1), has complete penetrance, and is quite healthy over TM6B.

In the initial screen, vials were scored as mutant if they had fewer than 25% as many deficiency heterozygote as balancer heterozygote flies. On retesting, this was revised to 50% of the level seen in control crosses. Balancer heterozygotes were approximately two-thirds as viable as deficiency heterozygotes.

In situ hybridization and cytological analysis

In situ hybridization of polytene chromosomes was carried out as described in Experimental Example I (see Methods, section A). Cytological analysis was performed by squashing larval salivary glands in lactoacetic orcein (2% orcein, 50% acetic acid, 30% lactic acid).

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the claims.

A

```
3242
TCG ATG GCC AGC TCG CAC TCG GTG CTG GCC AAG TCT CTC ATG GCC GAG CCG CGC
Ser MET Ala Ser Ser His Ser Val Leu Ala Lys Ser Leu MET Ala Glu Pro Arg
                                                                    972

ATG ACG CCC GAG CAG ATG AAG CGC AGC GAT ATT ATC CAA AAC TAC TTG AAG CGC
MET Thr Pro Glu Gln MET Lys Arg Ser Asp Ile Ile Gln Asn Tyr Leu Lys Arg

GAG AAC AGC ACA GCA GCC AGC AGC ACC ACC AAT GGC GTG GGC AAC CGC AGT CCC
Glu Asn Ser Thr Ala Ala Ser Ser Thr Thr Asn Gly Val Gly Asn Arg Ser Pro

AGC AGC AGC TCC ACA CCG CCG CCG TCG GCG GTC CAG AAT CAG CAG CGT TGG GGC
Ser Ser Ser Ser Thr Pro Pro Pro Ser Ala Val Gln Asn Gln Gln Arg Trp Gly
                                                                   1026

AGC AGC TCG GTG ATC ACC ACC ACC TGC CAG CAG CGC CAG CAG TCC GTG TCG CCG
Ser Ser Ser Val Ile Thr Thr Thr Cys Gln Gln Arg Gln Gln Ser Val Ser Pro

3512
CAC AGC AAC GGT TCC AGC TCC AGT TCG AGC TCT AGC TCC AGC TCC AGT TCG TCA
His Ser Asn Gly Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser

TCC TCC TCC ACA TCC TCC AAC TGC AGC TCC AGC TCG GCC AGC AGC TGC CAG TAT
Ser Ser Ser Thr Ser Ser Asn Cys Ser Ser Ser Ser Ala Ser Ser Cys Gln Tyr
                                                                   1080

TTC CAG TCG CCG CAC TCC ACC AGC AAC GGC ACC AGT GCA CCG GCG AGC TCC AGT
Phe Gln Ser Pro His Ser Thr Ser Asn Gly Thr Ser Ala Pro Ala Ser Ser Ser

TCG GGA TCG AAC AGC GCC ACG CCC CTG CTG GAA CTG CAG GTG GAC ATT GCT GAC
Ser Gly Ser Asn Ser Ala Thr Pro Leu Leu Glu Leu Gln Val Asp Ile Ala Asp

TCG GCG CAG CCT CTC AAT TTG TCC AAG AAA TCG CCC ACG CCG CCG CCC AGC AAG
Ser Ala Gln Pro Leu Asn Leu Ser Lys Lys Ser Pro Thr Pro Pro Pro Ser Lys
                                                                   1134
3782
CTG CAC GCT CTG GTG GCC GCC GCC AAT GCC GTT CAA AGG TAT CCC ACA TTG TCC
Leu His Ala Leu Val Ala Ala Ala Asn Ala Val Gln Arg Tyr Pro Thr Leu Ser

GCC GAC GTC ACA GTG ACA GCC TCC AAT GGC GGG TCC TCC GTC GGC GGC GGC GAG
Ala Asp Val Thr Val Thr Ala Ser Asn Gly Gly Ser Ser Val Gly Gly Gly Glu

TCC GGC CGC CAG CAG CAG TCC GCC GGC GAG TGT GGG CTC CCC CAA TCC GGG CCT
Ser Gly Arg Gln Gln Gln Ser Ala Gly Glu Cys Gly Leu Pro Gln Ser Gly Pro
                                                                   1186

GAG CGC CGC CGT GCA CAA GGT AAT GCT GGA GGC GTA AGA GCG GGA GGA GGT AGG
Glu Arg Arg Arg Ala Gln Gly Asn Ala Gly Gly Val Arg Ala Gly Gly Gly Arg

TGG TTT TAC GCG GAG AAG TGG GAG AGA CAG AGA CTG GGA GTG GCA GTT CAG CGA
Trp Phe Tyr Ala Glu Lys Trp Glu Arg Gln Arg Leu Gly Val Ala Val Gln Arg

4052
AGC AGG AAG CAG GAT CAC TTG GAG CGG CGG GAG TTG AAT TAA
Ser Arg Lys Gln Asp His Leu Glu Arg Arg Glu Leu Asn  .
                                                1237

[illegible]

4234
[illegible]

4374
[illegible]

4514
[illegible]

                              └─▸ poly A site  cDm927 and cDm926
4654
[illegible]

4794
[illegible]

4934
[illegible]

5074
[illegible]

5214
[illegible]

5354
[illegible]
   └─▸ putative polyadenylation signal for long transcripts
```

B

```
[illegible]
                              -141
[illegible]

                              +1
[illegible]
                              └─▸ mRNA start site

[illegible]

                              +211
[illegible]
                                             284
    [illegible]                              ATG GTT TGT GCA ATG CAA
                                             MET Val Cys Ala MET Gln
                                             1
302
CAG GTT GCT GCT GTG CAG CAT CAG CAG CAG CAA CAG CAA CTC CAG TTG CCC CAG
Gln Val Ala Ala Val Gln His Gln Gln Gln Gln Gln Gln Leu Gln Leu Pro Gln
                                                                     24

CAG CAA CAG CAG CAG CAG CAG ACA ACA CAG CAG CAA CAT GCA ACA ACG ATA GTC
Gln Gln Gln Gln Gln Gln Gln Thr Thr Gln Gln Gln His Ala Thr Thr Ile Val

CTG CTG ACG GGC AAT GGC GGC GGT AAT CTG CAC ATT GTC GCC ACA CCG CAA CAG
Leu Leu Thr Gly Asn Gly Gly Gly Asn Leu His Ile Val Ala Thr Pro Gln Gln

CAT CAG CCG ATG CAT CAG CTC CAC CAT CAG CAT CAG CAT CAG CAT CAG CAC CAG
His Gln Pro MET His Gln Leu His His Gln His Gln His Gln His Gln His Gln
                                                                     78

CAG CAG GCC AAG AGC CAA CAG CTG AAG CAA CAA CAC TCG GCG CTG GTC AAG TTG
Gln Gln Ala Lys Ser Gln Gln Leu Lys Gln Gln His Ser Ala Leu Val Lys Leu

572
CTG GAG TCG GCG CCC ATC AAG CAG CAA CAG CAG ACG CCC AAG CAA ATT GTT TAC
Leu Glu Ser Ala Pro Ile Lys Gln Gln Gln Gln Thr Pro Lys Gln Ile Val Tyr

CTG CAG CAG CAG CAG CAG CAA CCG CAA CGC AAA AGA CTG AAA AAC GAA GCA GCA
Leu Gln Gln Gln Gln Gln Gln Pro Gln Arg Lys Arg Leu Lys Asn Glu Ala Ala
                                                                    132

ATC GTA CAA CAG CAA CAA CAA ACA CCT GCA ACA CTA GTA AAG ACA ACA ACC ACC
Ile Val Gln Gln Gln Gln Gln Thr Pro Ala Thr Leu Val Lys Thr Thr Thr Thr

AGC AAC AGC AAC AGC AAC AAC ACC CAG ACA ACA AAT AGT ATT AGT CAG CAG CAA
Ser Asn Ser Asn Ser Asn Asn Thr Gln Thr Thr Asn Ser Ile Ser Gln Gln Gln

CAG CAG CAT CAG ATT GTG TTG CAG CAC CAG CAG CCA GCC GCG GCA GCA ACA CCA
Gln Gln His Gln Ile Val Leu Gln His Gln Gln Pro Ala Ala Ala Ala Thr Pro
                                                                    186
842
AAG CCA TGT GCC GAT CTG AGC GCC AAA AAT GAC AGC GAG TCG GGC ATC GAC GAG
Lys Pro Cys Ala Asp Leu Ser Ala Lys Asn Asp Ser Glu Ser Gly Ile Asp Glu

GAC TGC CCC AAC AGC GAT GAG GAT TGC CCC AAT GCC AAC CCG GCG GGC ACA TCG
Asp Cys Pro Asn Ser Asp Glu Asp Cys Pro Asn Ala Asn Pro Ala Gly Thr Ser

CTC GAG GAC AGC AGC TAC GAG CAG TAT CAG TGC CCC TGG AAG AAG ATA CGC TAT
Leu Glu Asp Ser Ser Tyr Glu Gln Tyr Gln Cys Pro Trp Lys Lys Ile Arg Tyr
                                                                    240

GCG CGT GAG CTC CTC AAG CAG CGC GAG TTG GAG CAG CAG CAG ACC ACC GGA GGC
Ala Arg Glu Leu Leu Lys Gln Arg Glu Leu Glu Gln Gln Gln Thr Thr Gly Gly

AGC AAC GCG CAG CAG CAA GTC GAG GCG AAG CCA GCT GCA ATA CCC ACC AGC AAC
Ser Asn Ala Gln Gln Gln Val Glu Ala Lys Pro Ala Ala Ile Pro Thr Ser Asn

1112
ATC AAG CAG CTG CAC TGT GAT AGT CCC TTT TCG GCG CAG ACC CAC AAG GAA ATC
Ile Lys Gln Leu His Cys Asp Ser Pro Phe Ser Ala Gln Thr His Lys Glu Ile
                                                                    294

GCC AAT CTC CTG CGC CAA CAG TCC CAG CAA CAA CAG GTT GTG GCC ACG CAG CAG
Ala Asn Leu Leu Arg Gln Gln Ser Gln Gln Gln Gln Val Val Ala Thr Gln Gln

CAG CAG CAA CAG CAG CAG CAG CAC CAG CAC CAG CAA CAA CGA AGG GAT AGC TCC
Gln Gln Gln Gln Gln Gln Gln His Gln His Gln Gln Gln Arg Arg Asp Ser Ser

GAC AGC AAC TGC TCG CTG ATG AGC AAC TCG AGC AAC TCC AGT GCG GGC AAT TGT
Asp Ser Asn Cys Ser Leu MET Ser Asn Ser Ser Asn Ser Ser Ala Gly Asn Cys
                                                                    348

TGC ACC TGC AAC GCT GGC GAC GAC CAG CAG CTG GAG GAG ATG GAC GAG GCC CAC
Cys Thr Cys Asn Ala Gly Asp Asp Gln Gln Leu Glu Glu MET Asp Glu Ala His

1382
GAT TCG GGC TGC GAC GAT GAA CTT TGC GAG CAG CAT CAC CAG CGA CTG GAC TCC
Asp Ser Gly Cys Asp Asp Glu Leu Cys Glu Gln His His Gln Arg Leu Asp Ser

TCC CAA CTG AAT TAC CTG TGC CAG AAG TTC GAT GAG AAA CTG GAC ACG GCG CTG
Ser Gln Leu Asn Tyr Leu Cys Gln Lys Phe Asp Glu Lys Leu Asp Thr Ala Leu
                                                                    402

AGC AAC AGC AGC GCC AAC ACG GGG AGG AAC ACG CCA GCT GTA ACA GCT AAC GAA
Ser Asn Ser Ser Ala Asn Thr Gly Arg Asn Thr Pro Ala Val Thr Ala Asn Glu

1544
GAT GCC GAT [illegible]
Asp Ala Asp
```

A

ACTTACTAGTGAAAAACATGATAATAAACAACTTCCCAAAAAAAATCCAATGAAATTGACA

CTTATGTTAAAAAAATAGGTGAGATTGTAACCGTTGATGTACACTTACGAAGTACGTAACAAGTTCATGA

                                    -141
ACTGATTTCGTGAGCAGGTCTCTCCATAATCGCCGTATCTGTGGGATCGCGCGCTCCTGCTCGCACTCCC

TGGGTGGATGGCAGCACATGTTCGAAGTCGGAGACAGTGCAAAGCGGAGAGCGCCGACGTCGACGCCGAA

                                    -1
AAAACTGAACAAGATCCGCCGCGAATGTTGATTTTTCCTTTCATTGACTAACTGCCACTCGCAGCGCGCAG

                         ┌───→ mRNA start site

ATCGTCGGCTCCGGCTTGTTCGCGTTCCGTTCGTTTCGTTTCGTTTCGTTCGATCTACTTCGAGTCGCGAGT

TTTAAGCAGTGTAGTGAGTGCCCCCGTGAAAAGGATAACCCAAAAAGTGATTTCTACTATTTTCCAATAGT

                                    +211
TTTTATCAGTGTGAAGAAAACATGTAAACTTGGCTCAAAAAAGGGCTTTAAAAGATACAAAGCTTCAATGC

GAAGGATAAAATAATATCGCACCAGTGCTTCAAAAAACCAAAACTATGCCTAAGGCTGGAAATTTAAATTA

AAATTTTTTTAATAAATATTCCAAAAATATTGCCCCTGAAAAGTGTTGATAAACCCCAACCGAGCAAA

380
ATG TTA ATG TCC GCG GAC AGT TCA GAT AGC GCC AAG ACT TCT GTG ATC TGC AGC
MET Leu MET Ser Ala Asp Ser Ser Asp Ser Ala Lys Thr Ser Val Ile Cys Ser

ACG GTG AGT GCC AGC ATG CTA GCA CCA CCA GCT CCA GAA CAG CCC AGC ACC ACA
Thr Val Ser Ala Ser MET Leu Ala Pro Pro Ala Pro Glu Gln Pro Ser Thr Thr

GCA CCA CCC ATT TTG GGG GTA ACA GGT CGA TCT CAC CTG GAA AAT GCC CTG AAA
Ala Pro Pro Ile Leu Gly Val Thr Gly Arg Ser His Leu Glu Asn Ala Leu Lys
                                                                      54
542
CTA CCG CCA AAC ACA AGT GTT TCG GCT TAC TAC CAG CAC AAC AGC AAG CTG GGC
Leu Pro Pro Asn Thr Ser Val Ser Ala Tyr Tyr Gln His Asn Ser Lys Leu Gly

ATG GGC CAG AAT TAC AAT CCG GAA TTC AGG AGC CTG GTA GCA CCT GTC ACA GAT
MET Gly Gln Asn Tyr Asn Pro Glu Phe Arg Ser Leu Val Ala Pro Val Thr Asp

CTG GAT ACT GTG CCA CCC ACA GGT GTG ACC ATG GCG AGT TCT TCG AAT TCT CCC
Leu Asp Thr Val Pro Pro Thr Gly Val Thr MET Ala Ser Ser Ser Asn Ser Pro
                                                                      108
AAC TCC TCC GTC AAG CTG CCC CAC AGC GGC GTG ATC TTT GTC AGC AAA TCG AGT
Asn Ser Ser Val Lys Leu Pro His Ser Gly Val Ile Phe Val Ser Lys Ser Ser

GCC GTC AGC ACC ACC GAT GGT CCC ACT GCA GTG TTG CAA CAG CAG CAG CCG CAG
Ala Val Ser Thr Thr Asp Gly Pro Thr Ala Val Leu Gln Gln Gln Gln Pro Gln

812
CAG CAA ATG CCC CAG CAC TTC GAG TCC CTG CCC CAC CAC CAC CCC CAG CAG GAA
Gln Gln MET Pro Gln His Phe Glu Ser Leu Pro His His His Pro Gln Gln Glu
                                                                      162

812
CAG CAA ATG CCC CAG CAC TTC GAG TCC CTG CCC CAC CAC CAC CCC CAG CAG GAA
Gln Gln MET Pro Gln His Phe Glu Ser Leu Pro His His His Pro Gln Gln Glu
                                                                    162

CAC CAG CCA CAG CAG CAG CAG CAA CAA CAT CAC CTT CAG CAC CAC CCA CAT CCA
His Gln Pro Gln Gln Gln Gln Gln Gln His His Leu Gln His His Pro His Pro

CAT GTG ATG TAT CCG CAC GGA TAT CAG CAG GCC AAT CTG CAC CAC TCG GGT GGT
His Val MET Tyr Pro His Gly Tyr Gln Gln Ala Asn Leu His His Ser Gly Gly

ATT GCT GTG GTT CCG GCG GAT TCG CGT CCC CAG ACT CCC GAG TAC ATC AAG TCC
Ile Ala Val Val Pro Ala Asp Ser Arg Pro Gln Thr Pro Glu Tyr Ile Lys Ser
                                                                    216

TAC CCA GTT ATG GAT ACA ACT GTG GCT AGT TCG GTA AAG GGG GAA CCA GAA CTC
Tyr Pro Val MET Asp Thr Thr Val Ala Ser Ser Val Lys Gly Glu Pro Glu Leu

GTGAGTTGTG..intron 1..TTCTTTGCAG

1082
AAC ATA GAA TTC GAT GGC ACC ACA GTG CTG TGC CGC GTT TGC GGG GAT AAG GCC
Asn Ile Glu Phe Asp Gly Thr Thr Val Leu Cys Arg Val Cys Gly Asp Lys Ala

                              GTAAGTTCGT..intron 2..ATCGTTTCAG

TCC CGT TTC CAT TAC GGC GTG CAT TCC TGC GAG GGT TGC AAG GGA TTC TTC CGC
Ser Gly Phe His Tyr Gly Val His Ser Cys Glu Gly Cys Lys Gly Phe Phe Arg
                                                                    270

CGC TCC ATC CAG CAA AAG ATC CAG TAT CGC CCG TGC ACC AAG AAT CAG CAG TGC
Arg Ser Ile Gln Gln Lys Ile Gln Tyr Arg Pro Cys Thr Lys Asn Gln Gln Cys

AGC ATT CTG CGC ATC AAT CGC AAT CGT TGT CAA TAT TGC CGC CTG AAA AAG TGC
Ser Ile Leu Arg Ile Asn Arg Asn Arg Cys Gln Tyr Cys Arg Leu Lys Lys Cys

                  GTGAGTACCT..intron 3..CCAATTGCAG

ATT GCC GTG GGC ATG AGT CGC GAT GCT GTG CGT TTT GGA CGC GTG CCG AAG CGC
Ile Ala Val Gly MET Ser Arg Asp Ala Val Arg Phe Gly Arg Val Pro Lys Arg
                                                                    324

1352
GAA AAG GCG CGT ATC CTG GCG GCC ATG CAA CAG AGC ACC CAG AAT CGC GGC CAG
Glu Lys Ala Arg Ile Leu Ala Ala MET Gln Gln Ser Thr Gln Asn Arg Gly Gln

CAG CGA GCC CTC GCC ACC GAG CTG GAT GAC CAG CCA CGC CTC CTC GCC GCC GTG
Gln Arg Ala Leu Ala Thr Glu Leu Asp Asp Gln Pro Arg Leu Leu Ala Ala Val

CTG CGC GCC CAC CTC GAG ACC TGT GAG TTC ACC AAG GAG AAG GTC TCG GCG ATG
Leu Arg Ala His Leu Glu Thr Cys Glu Phe Thr Lys Glu Lys Val Ser Ala MET
                                                                    378

                              GTAAGTCTCA..intron 4..ATTTCTTCAG

CGG CAG CGG GCG CGG GAT TGC CCC TCC TAC TCC ATG CCC ACA CTT CTG GCC TGT
Arg Gln Arg Ala Arg Asp Cys Pro Ser Tyr Ser MET Pro Thr Leu Leu Ala Cys

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CCG CTG AAC CCC GCC CCT GAA CTG CAA TCG GAG CAG GAG TTC TCC CAG CCT TTC
Pro Leu Asn Pro Ala Pro Glu Leu Gln Ser Glu Gln Glu Phe Ser Gln Arg Phe
1622
GCC CAC GTA ATT CGC GGC GTG ATC GAC TTT GCC GGC ATG ATT CCC GGC TTC CAG
Ala His Val Ile Arg Gly Val Ile Asp Phe Ala Gly MET Ile Pro Gly Phe Gln
                                                                      432
CTG CTC ACC CAG GAC GAT AAG TTC ACG CTC CTG AAG GCG GGA CTC TTC GAC GCC
Leu Leu Thr Gln Asp Asp Lys Phe Thr Leu Leu Lys Ala Gly Leu Phe Asp Ala

CTG TTT GTG CGC CTG ATC TGC ATG TTT GAC TCG TCG ATA AAC TCA ATC ATC TGT
Leu Phe Val Arg Leu Ile Cys MET Phe Asp Ser Ser Ile Asn Ser Ile Ile Cys

CTA AAT GGC CAG GTG ATG CGA CGG GAT GCG ATC CAG AAC GGA GCC AAT GCC CGC
Leu Asn Gly Gln Val MET Arg Arg Asp Ala Ile Gln Asn Gly Ala Asn Ala Arg
                                                                      486
TTC CTG GTG GAC TCC ACC TTC AAT TTC GCG GAG CGC ATG AAC TCG ATG AAC CTG
Phe Leu Val Asp Ser Thr Phe Asn Phe Ala Glu Arg MET Asn Ser MET Asn Leu
1892
ACA GAT GCC GAG ATA GGC CTG TTC TGC GCC ATC GTT CTG ATT ACG CCG GAT CGC
Thr Asp Ala Glu Ile Gly Leu Phe Cys Ala Ile Val Leu Ile Thr Pro Asp Arg

CCC GGT TTG CGC AAC CTG GAG CTG ATC GAG AAG ATG TAC TCG CGA CTC AAG GGC
Pro Gly Leu Arg Asn Leu Glu Leu Ile Glu Lys MET Tyr Ser Arg Leu Lys Gly
                                                                      540
TGC CTG CAG TAC ATT GTC GCC CAG AAT AGG CCC GAT CAG CCC GAG TTC CTG GCC
Cys Leu Gln Tyr Ile Val Ala Gln Asn Arg Pro Asp Gln Pro Glu Phe Leu Ala

AAG TTG CTG GAG ACG ATG CCC GAT CTG CGC ACC CTG AGC ACC CTG CAC ACC GAG
Lys Leu Leu Glu Thr MET Pro Asp Leu Arg Thr Leu Ser Thr Leu His Thr Glu

AAA CTG GTA GTT TTC CGC ACC GAG CAC AAG GAG CTG CTG CGC CAG CAG ATG TGG
Lys Leu Val Val Phe Arg Thr Glu His Lys Glu Leu Leu Arg Gln Gln MET Trp
2162                                                                  594
TCC ATG GAG GAC GGC AAC AAC AGC GAT GGC CAG CAG AAC AAG TCG CCC TCG GGC
Ser MET Glu Asp Gly Asn Asn Ser Asp Gly Gln Gln Asn Lys Ser Pro Ser Gly

AGC TGG GCG GAT GCC ATG GAC GTG GAG GCG GCC AAG AGT CCG CTT GGC TCG GTA
Ser Trp Ala Asp Ala MET Asp Val Glu Ala Ala Lys Ser Pro Leu Gly Ser Val

TCG AGC ACT GAG TCC GCC GAC CTG GAC TAC GGC AGT CCG AGC AGT TCG CAG CCA
Ser Ser Thr Glu Ser Ala Asp Leu Asp Tyr Gly Ser Pro Ser Ser Ser Gln Pro
                                                                      648
CAG GGC GTG TCT CTG CCC TCG CCG CCT CAG CAA CAG CCC TCG CCT CTG GCC AGC
Gln Gly Val Ser Leu Pro Ser Pro Pro Gln Gln Gln Pro Ser Ala Leu Ala Ser

TCG GCT CCT CTG CTG GCG GCC ACC CTC TCC GGA GGA TGT CCC CTG CGC AAC CGG
Ser Ala Pro Leu Leu Ala Ala Thr Leu Ser Gly Gly Cys Pro Leu Arg Asn Arg
2432
GCC AAT TCC GGC TCC ACC GGT GAC TCC GGA GCA GCT GAG ATG GAT ATC GTT GGC
Ala Asn Ser Gly Ser Ser Gly Asp Ser Gly Ala Ala Glu MET Asp Ile Val Gly
                                                                      702
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2432
GCC AAT TCC GGC TCC AGC GGT GAC TCC GGA CCA GCT GAG ATG GAT ATC GTT GGC
Ala Asn Ser Gly Ser Ser Gly Asp Ser Gly Ala Ala Glu MET Asp Ile Val Gly
                                                                      702

TCG CAC GCA CAT CTC ACC CAG AAC GGG CTG ACA ATC ACG CCG ATT GTG CGA CAC
Ser His Ala His Leu Thr Gln Asn Gly Leu Thr Ile Thr Pro Ile Val Arg His

                    GTAGTATCTT..intron 5..TTTCTTACAG

CAG CAG CAG CAA CAA CAG CAG CAG CAG ATC GGA ATA CTC AAT AAT GCG CAT TCC
Gln Gln Gln Gln Gln Gln Gln Gln Gln Ile Gly Ile Leu Asn Asn Ala His Ser

CGC AAC TTG AAT GGG GGA CAC GCG ATG TGC CAG CAA CAG CAG CAG CAC CCA CAA
Arg Asn Leu Asn Gly Gly His Ala MET Cys Gln Gln Gln Gln Gln His Pro Gln
                                                                      756
                                                G (Dm4 925)
CTG CAC CAC CAC TTG ACA GCC GGA GCT GCC CGC TAC AGA AAG CTA GAT TCG CCC
Leu His His His Leu Thr Ala Gly Ala Ala Arg Tyr Arg Lys Leu Asp Ser Pro
                                                          Arg
2702
ACG GAT TCG GGC ATT GAG TCG GGC AAC GAG AAG AAC GAG TGC AAG GCG GTG AGT
Thr Asp Ser Gly Ile Glu Ser Gly Asn Glu Lys Asn Glu Cys Lys Ala Val Ser

TCG GGG GGA AGT TCC TCG TGC TCC AGT CCG CGT TCC AGT GTG GAT GAT GCG CTG
Ser Gly Gly Ser Ser Ser Cys Ser Ser Pro Arg Ser Ser Val Asp Asp Ala Leu
                                                                      810

GAC TGC AGC GAT GCC GCC GCC AAT CAC AAT CAG GTG GTG CAG CAT CCG CAG CTG
Asp Cys Ser Asp Ala Ala Ala Asn His Asn Gln Val Val Gln His Pro Gln Leu

AGT GTG GTG TCC GTG TCA CCA GTT CGC TCG CCC CAG CCC TCC ACC AGC AGC CAT
Ser Val Val Ser Val Ser Pro Val Arg Ser Pro Gln Pro Ser Thr Ser Ser His

CTG AAG CGA CAG ATT GTG GAG GAT ATG CCC GTG CTG AAG CGC GTG CTG CAG GCT
Leu Lys Arg Gln Ile Val Glu Asp MET Pro Val Leu Lys Arg Val Leu Gln Ala
                                                                      864
2972
CCC CCT CTG TAC GAT ACC AAC TCG CTG ATG GAC GAG GCC TAC AAG CCG CAC AAG
Pro Pro Leu Tyr Asp Thr Asn Ser Leu MET Asp Glu Ala Tyr Lys Pro His Lys

AAA TTC CGG GCC CTG CGG CAT CGC GAG TTC GAG ACC GCC GAG GCG GAT GCC AGC
Lys Phe Arg Ala Leu Arg His Arg Glu Phe Glu Thr Ala Glu Ala Asp Ala Ser

AGT TCC ACT TCC GGC TCG AAC AGC CTG AGT GCC GCC AGT CCG CGG CAG AGC CCA
Ser Ser Thr Ser Gly Ser Asn Ser Leu Ser Ala Gly Ser Pro Arg Gln Ser Pro
                                                                      918

GTC CCG AAC AGT GTG GCC ACG CCC CCG CCA TCG GCG GCC AGC GCC GCC GCA GGT
Val Pro Asn Ser Val Ala Thr Pro Pro Pro Ser Ala Ala Ser Ala Ala Ala Gly

AAT CCC GCC CAG AGC CAG CTG CAC ATG CAC CTG ACC CGC AGC AGC CCC AAG GCC
Asn Pro Ala Gln Ser Gln Leu His MET His Leu Thr Arg Ser Ser Pro Lys Ala

3242
TCG ATG GCC AGC TCG CAC TCG GTG CTG GCC AAG TCT CTC ATG GCC GAG CCG CGC
Ser MET Ala Ser Ser His Ser Val Leu Ala Lys Ser Leu MET Ala Glu Pro Arg
                                                                        972

ATG ACG CCC GAG CAG ATG AAG CCC AGC GAT ATT ATC CAA AAC TAC TTG AAG CGC
MET Thr Pro Glu Gln MET Lys Arg Ser Asp Ile Ile Gln Asn Tyr Leu Lys Arg

GAG AAC AGC ACA GCA GCC AGC AGC ACC ACC AAT GGC GTG GGC AAC CGC AGT CCC
Glu Asn Ser Thr Ala Ala Ser Ser Thr Thr Asn Gly Val Gly Asn Arg Ser Pro

AGC AGC AGC TCC ACA CCG CCG CCG TCG GCG GTC CAG AAT CAG CAG CGT TGG GGC
Ser Ser Ser Ser Thr Pro Pro Pro Ser Ala Val Gln Asn Gln Gln Arg Trp Gly
                                                                        1026

AGC AGC TCG GTG ATC ACC ACC ACC TGC CAG CAG CGC CAG CAG TCC GTG TCG CCG
Ser Ser Ser Val Ile Thr Thr Thr Cys Gln Gln Arg Gln Gln Ser Val Ser Pro

3512
CAC AGC AAC GGT TCC AGC TCC AGT TCG AGC TCT AGC TCC AGC TCC AGT TCG TCA
His Ser Asn Gly Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser

TCC TCC TCC ACA TCC TCC AAC TGC AGC TCC AGC TCG GCC AGC AGC TGC CAG TAT
Ser Ser Ser Thr Ser Ser Asn Cys Ser Ser Ser Ser Ala Ser Ser Cys Gln Tyr
                                                                        1080

TTC CAG TCG CCG CAC TCC ACC AGC AAC GGC ACC AGT GCA CCG GCG AGC TCC AGT
Phe Gln Ser Pro His Ser Thr Ser Asn Gly Thr Ser Ala Pro Ala Ser Ser Ser

TCG GGA TCG AAC AGC GCC ACG CCC CTG CTG GAA CTG CAG GTG GAC ATT GCT GAC
Ser Gly Ser Asn Ser Ala Thr Pro Leu Leu Glu Leu Gln Val Asp Ile Ala Asp

TCG GCG CAG CCT CTC AAT TTG TCC AAG AAA TCG CCC ACG CCG CCG CCC ACC AAG
Ser Ala Gln Pro Leu Asn Leu Ser Lys Lys Ser Pro Thr Pro Pro Pro Ser Lys
                                                                        1134
3782
CTG CAC GCT CTG GTG GCC GCC GCC AAT GCC GTT CAA AGG TAT CCC ACA TTG TCC
Leu His Ala Leu Val Ala Ala Ala Asn Ala Val Gln Arg Tyr Pro Thr Leu Ser

GCC GAC GTC ACA GTG ACA GCC TCC AAT GGC GGG TCC TCC GTC GGC GGC GGC GAG
Ala Asp Val Thr Val Thr Ala Ser Asn Gly Gly Ser Ser Val Gly Gly Gly Glu

TCC GGC CGC CAG CAG CAG TCC GCC GGC GAG TGT GGG CTC CCC CAA TCC GGG CCT
Ser Gly Arg Gln Gln Gln Ser Ala Gly Glu Cys Gly Leu Pro Gln Ser Gly Pro
                                                                        1186

GAG CGC CGC CGT GCA CAA GGT AAT GCT GGA GGC GTA AGA GCG GGA GGA GGT AGG
Glu Arg Arg Arg Ala Gln Gly Asn Ala Gly Gly Val Arg Ala Gly Gly Gly Arg

TGG TTT TAC GCG GAG AAG TGG GAG AGA CAG AGA CTG GGA GTG GCA GTT CAG CGA
Trp Phe Tyr Ala Glu Lys Trp Glu Arg Gln Arg Leu Gly Val Ala Val Gln Arg

4052
AGC AGG AAG CAG GAT CAC TTG GAG CGG CGG GAG TTG AAT TAA
Ser Arg Lys Gln Asp His Leu Glu Arg Arg Glu Leu Asn   .
                                                        1237

4052
AGC AGG AAG CAG GAT CAC TTG GAG CGG CGG GAG TTG AAT TAA
Ser Arg Lys Gln Asp His Leu Glu Arg Arg Glu Leu Asn
1237

ATTATTTTACCATTTAATTGAGACGTGTACAAAGTTTGAAAGCAAAACCAACATGCATGCAATTTAAAAC

TAATATTTAAAGCAACAACAAACAAAACAACTACAAGTTATTAATTTAAAAAACAAACAAACAAACAAAC

4234
AACAAAAAACCCAAGCTTGAATGGTATTACAAAAGAAAAAGAAAAACAGAAAAAATATAAATATATTTTA

CCAGTTAAACTTTAACGTAGCAAGAAACCAACAAACCCAAGGCAGCGCTCTGATTTCGCATTAACTTTTC

4374
TTCAGCTGCTACGGAAAACGCCCCTCACCTCCCCCCCACCCAACCCTTCCTCCACACACCAACCGTCTTT

CGACCCCTGATTGTTTTATAAGTTTTAAGCTCTTGTTGTACATATTAATTACGTTTATTGGTAACTATGT

4514
TTAGCGCTTTAGTTGTAGTTCGAGCAAAACTACTTTCCTTTTTTCGATGTTTTTTGAAAAAACTGCAAAT

TATTATT<u>ATTAAA</u>TTTTTAAATACCTAAAAACAAAACAATGTGTGTGAAATTTTTTATTGTCCGATCTCC

⌐→ poly A site cDm4927 and cDm4928

4654
AAGCAGAATGAAGTGCAGTTTGCAACAAATTTTAACTACGATTAAGTTGATAACGATTCATTTTTTATGA

ATTTAACTAATTTTATGAATTTGTTATAGTTTTTCCACCCTTCTATAGATCTTCTATCTGATCATCTAGCT

4794
ACCCGTATTCCTCATTTCTCCTTTGGCACAAAGCTCTTCTCTATGCTAAAGAATCAAGTGGAATAAATAT

TGTTTTCTAATTTTAAAACTACCACAAAAATACGATTAAAAATATACACGAAGTAATCAAAATCAAACAAA

4934
ATGCTTAAAGTTTTAGCAGCAACCAGTAAAACGACGATCAAGAAGAGAAACCCAACGTTAAATATATCTG

TTGTGTACATAGTTAAATGTTAAATTAAACACAAAAACATATTTAAAGTACATATAAATACACATAATTA

5074
TTAATGAAGAAACCTATGCTTAAAAGATTCAATGTTTGATTGCCATCTTAGAAAACCAAGCGAAAAATAC

AAAAAAAAATCAACAAACAAAAATTATGATATATTATTTAAAAGTAAAGTATACATTTACATTACAGAAA

5214
AACAAAAGAGAAAACTTGCGGTAGCAACAAAACTATTATATTAATTACATTTTAATTATGCTGTACTATT

ATGATTATTAATTATTATGATTAATTAATTACGATTTTTATGCTTAGACAAACCAACAAAAAACAAATAT

5354
GCAAAAACC<u>ATTAAA</u>AAAAAAAAACAAAAAACAAGCAAAAAAT

⌐→ putative polyadenylation signal for long transcripts

B

CGACCCGTTTCGAGTGAACGTCCTCAGTTCGCACACAAAAACAAAAACACAAAACCACAGCAACAACATC

−141
CGTGCGCGGAGTACCAGCGGGATGGGGTAATGGGGGGCACCGCGGGGAGTGGAGCCCGAGAGACGAACAG

ACGGACCCGAAGCAACACAACACCAACACGAGCGCCCAAAAAGACACTTCCGCTGCGTTCAGCTCCTGTTG

−1
CTCTCGGTCGTTTTTGTAATGCTCGTCGACCGTCCTTTCATTCGCAAATTGCTCGTCGTTGCCAGCGGGTTG

                              1
                              ⌐⟶ mRNA start site

TGCAGAGCAAGAAAAGCGCGCGAAAAACCAAGCAAAAAATTAATACAGCTGGATCAAGCGAAAGAGATAG

AGAGCAGAGTCAACAGCAACAAATGTTCAATAGCAAATCATATCGCATATTTTTGTTGGTGCCAGTGAAG

+211
TGAGATCAAAGTGAAGTGTGCAATGTTCCTTATTAGCAAATCGTAGAGCAACCAACAATCGAGAGTTCAA

                                   284
       GTGTCATTTCGAAGCCAAAAAGCAAAATCTCTAATTCAAAT ATG GTT TGT GCA ATG CAA
                                           MET Val Cys Ala MET Gln
                                           1

302
GAG GTT GCT GCT GTG CAG CAT CAG CAG CAG CAA CAG CAA CTC CAG TTG CCC CAG
Glu Val Ala Ala Val Gln His Gln Gln Gln Gln Gln Leu Gln Leu Pro Gln
                                                     24

CAG CAA CAG CAG CAG CAG CAG ACA ACA CAG CAG CAA CAT GCA ACA ACG ATA GTG
Gln Gln Gln Gln Gln Gln Gln Thr Thr Gln Gln Gln His Ala Thr Thr Ile Val

CTG CTG ACG GGC AAT GGC GCC GGT AAT CTG CAC ATT GTC GCC ACA CCG CAA CAG
Leu Leu Thr Gly Asn Gly Gly Gly Asn Leu His Ile Val Ala Thr Pro Gln Gln

CAT CAG CCG ATG CAT CAG CTC CAC CAT CAG CAT CAG CAT CAG CAT CAG CAC CAG
His Gln Pro MET His Gln Leu His His Gln His Gln His Gln His Gln His Gln
                                                       78

CAG CAG GCC AAG AGC CAA CAG CTG AAG CAA CAA CAC TCG GCG CTG GTC AAG TTG
Gln Gln Ala Lys Ser Gln Gln Leu Lys Gln Gln His Ser Ala Leu Val Lys Leu

572
CTG GAG TCG GCG CCC ATC AAG CAG CAA CAG CAG ACG CCC AAG CAA ATT GTT TAC
Leu Glu Ser Ala Pro Ile Lys Gln Gln Gln Gln Thr Pro Lys Gln Ile Val Tyr

CTG CAG CAG CAG CAG CAG CAA CCG CAA CCC AAA AGA CTG AAA AAC GAA CCA CCA
Leu Gln Gln Gln Gln Gln Gln Pro Gln Arg Lys Arg Leu Lys Asn Glu Ala Ala
                                                     132

ATC GTA CAA CAG CAA CAA CAA ACA CCT GCA ACA CTA GTA AAG ACA ACA ACC ACC
Ile Val Gln Gln Gln Gln Gln Thr Pro Ala Thr Leu Val Lys Thr Thr Thr Thr

ACC AAC ACC AAC AGC AAC AAC ACC CAG ACA ACA AAT AGT ATT AGT CAG CAG CAA
Ser Asn Ser Asn Ser Asn Asn Thr Gln Thr Thr Asn Ser Ile Ser Gln Gln Gln

AGC AAC AGC AAC AGC AAC AAC ACC CAG ACA ACA AAT AGT ATT AGT CAG CAG CAA
Ser Asn Ser Asn Ser Asn Asn Thr Gln Thr Thr Asn Ser Ile Ser Gln Gln Gln

CAG CAG CAT CAG ATT GTG TTG CAG CAC CAG CAG CCA GCC GCG GCA CCA ACA CCA
Gln Gln His Gln Ile Val Leu Gln His Gln Gln Pro Ala Ala Ala Ala Thr Pro
                                                                      186
842
AAG CCA TGT GCC GAT CTG AGC GCC AAA AAT GAC AGC GAG TCG GGC ATC GAC GAG
Lys Pro Cys Ala Asp Leu Ser Ala Lys Asn Asp Ser Glu Ser Gly Ile Asp Glu

GAC TCC CCC AAC AGC GAT GAG GAT TGC CCC AAT GCC AAC CCG GCG GGC ACA TCG
Asp Cys Pro Asn Ser Asp Glu Asp Cys Pro Asn Ala Asn Pro Ala Gly Thr Ser

CTC GAG GAC AGC AGC TAC GAG CAG TAT CAG TGC CCC TGG AAG AAG ATA CGC TAT
Leu Glu Asp Ser Ser Tyr Glu Gln Tyr Gln Cys Pro Trp Lys Lys Ile Arg Tyr
                                                                      240

GCG CGT GAG CTC CTC AAG CAG CGC GAG TTG GAG CAG CAG CAG ACC ACC GGA GGC
Ala Arg Glu Leu Leu Lys Gln Arg Glu Leu Glu Gln Gln Gln Thr Thr Gly Gly

AGC AAC GCG CAG CAG CAA GTC GAG GCG AAG CCA GCT GCA ATA CCC ACC AGC AAC
Ser Asn Ala Gln Gln Gln Val Glu Ala Lys Pro Ala Ala Ile Pro Thr Ser Asn

1112
ATC AAG CAG CTG CAC TGT GAT AGT CCC TTT TCG GCG CAG ACC CAC AAG GAA ATC
Ile Lys Gln Leu His Cys Asp Ser Pro Phe Ser Ala Gln Thr His Lys Glu Ile
                                                                      294

GCC AAT CTG CTG CGC CAA CAG TCC CAG CAA CAA CAG GTT GTG GCC ACG CAG CAG
Ala Asn Leu Leu Arg Gln Gln Ser Gln Gln Gln Gln Val Val Ala Thr Gln Gln

CAG CAG CAA CAG CAG CAG CAG CAC CAG CAC CAG CAA CAA CGA AGG GAT AGC TCC
Gln Gln Gln Gln Gln Gln Gln His Gln His Gln Gln Gln Arg Arg Asp Ser Ser

GAC AGC AAC TGC TCG CTG ATG AGC AAC TCG ACC AAC TCC AGT GCG GGC AAT TGT
Asp Ser Asn Cys Ser Leu MET Ser Asn Ser Ser Asn Ser Ser Ala Gly Asn Cys
                                                                      348

TGC ACC TGC AAC GCT GCC GAC GAC CAG CAG CTG GAG GAG ATG GAC GAG GCC CAC
Cys Thr Cys Asn Ala Gly Asp Asp Gln Gln Leu Glu Glu MET Asp Glu Ala His

1382
GAT TCG GGC TGC CAC GAT GAA CTT TGC GAG CAG CAT CAC CAG CGA CTG GAC TCC
Asp Ser Gly Cys Asp Asp Glu Leu Cys Glu Gln His His Gln Arg Leu Asp Ser

TCC CAA CTG AAT TAC CTG TGC CAG AAG TTC GAT GAG AAA CTG GAC ACG GCG CTG
Ser Gln Leu Asn Tyr Leu Cys Gln Lys Phe Asp Glu Lys Leu Asp Thr Ala Leu
                                                                      402

AGC AAC ACC ACC GCC AAC ACG GGG AGG AAC ACG CCA GCT GTA ACA GCT AAC GAA
Ser Asn Ser Ser Ala Asn Thr Gly Arg Asn Thr Pro Ala Val Thr Ala Asn Glu

1544
GAT GCC GAT gtaggtttag
Asp Ala Asp

EcR cDA　　1534 bp

Hck cDNA   5536 bp

(The body of this page is a rotated nucleotide sequence listing with corresponding amino acid translation, numbered by nucleotide position at the left margin (1, 125, 249, 373, 497, 621, 745, 869, 993, 1105, 1198, 1291, 1384, 1477, 1570, 1663, 1756, 1849, 1942, 2035, 2128, 2221, 2314, 2407) and by residue number at the right margin (12, 43, 74, 105, 136, 167, 198, 229, 260, 291, 322, 353, 384, 415, 446, 477).)

cDNA DBR3-9   4.2 kb

```
1     GAATCATTCAACTGCAANGCTGCCAANTCGCATNCGGGCATNCCGGGGNGCCGNGCATNGGCGGNTGCATACCGGTNCATACTACACGCTACAA
125   TTCCAACTATTTCACCAATCACANCGGCANCGGGCANCGGGCANCGGGCANCGGGNCGGCCAANCTTCGNTNGAANCCCTNGAANCCTTGNTACTAATAATT    ATG TAT ACG CAA CGT      5
                                                                                                      MET Tyr Thr Gln Arg

242   ATG TTT GAC ATG TGG TCC ACT TCG AAA CTG GGA GCA GAC CAC AAT CTC CGT CAA AAC AAC GTC CAA TCG CCG GGA CAA AAC     36
      MET Phe Asp MET Trp Ser Ser Val Thr Ser Lys Leu Gly Ala His Asn Leu Arg Gln Asn Asn Val Gln Ser Pro Gly Gln Asn
                  (gt-aag..v..tcacg)

335   AAC TCC ACC GGT TCC ATT AAA GCT CAA ATT GAG ATA ATT CCA TCG AAA GTC GCC GAC TCA TCC GGT GAT TAC GGA ATC ACC     67
      Asn Ser Ser Gly MET Ser Ile Lys Ala Gln Ile Glu Ile Ile Pro Ser Lys Val Gly Asp Ser Ser Gly Asp Tyr Gly Ile Thr

428   TGC GAG AAC GGA TCC AAG GGA TTC TTT CGA AGA TCG CAA AGC TAC TAC CAA ACG GTG GAC TGT GTG GAC GAC CGT GTT     98
      Cys Glu Asn Gly Ser Lys Gly Phe Phe Arg Arg Ser Gln Ser Tyr Tyr Gln Thr Val Asp Cys Val Asp Asp Arg Val

521   AAT CCC AAC CGA TGT CAA AAG TTC CTA AAA CTG GGA AAG ACC CGT GAT CCT GTA AAG TTC GCC AGG ATG TCC AAG AAG     129
      Asn Pro Asn Arg Cys Gln Lys Phe Leu Lys Leu Gly Lys Thr Arg Asp Pro Val Lys Phe Ala Arg MET Ser Lys Lys
                                                                           (gtacg..v..ttgcag)

614   CAG CCG GAG AAG GTC GAC TTC CAT CCG GCA CAA ACC CGA ATG GCC GCA CAA GCA GAT ACC TCC GTA TAC GAC ACA     160
      Gln Arg Glu Glu Val Asp Phe His Pro Ala Gln Thr Arg MET Ala Ala Gln Ala Asp Ser Val Tyr Asp Thr
                                                          (gt-acg..v..cctcag)

707   CAG CAC CCC TCG ACC CAC CAG CTG CTG AAC AAT TAC AAC ACC TAC AAC AAC GGG TAC GGC TAC GCC ACT     191
      Gln Thr Pro Ser Thr His Gln Leu Leu Asn Asn Tyr Asn Thr Tyr Asn Asn Gly Tyr Gly Tyr Ala Ser

800   TAC GGA TAC TGG GCC GTG AGG CCA ACG ATG CAG CAG TAC ATC TCG GCG CAG TAC ACC TAC GAC CGG ACT     222
      Tyr Gly Tyr Trp Ala Val Arg Pro Thr MET Gln Gln Tyr Ile Ser Ala Gln Tyr Thr Tyr Asp Arg Ser
                                           (gtacg..v..gtccag)    (C)

893   ACA ATA ATC GAT TTT ATT ACT CAC GCG GAT ATA AAC GGC GAT GTG CTG GTG ATC AAG ACG GCG AAC ACA AAT     253
      Thr Ile Ile Asp Phe Ile Thr His Ala Asp Ile Asn Gly Asp Val Leu Val Ile Lys Thr Ala Asn Thr Asn
                                                    (gtgcg..v..gtcacag)     (G)

986   ACC AAA CTG GCT GTG TTC CGA AAG CAG CCG GAT GTG TCA GCC ATT CTC TAC TAC AAG AAT CTG GAG GAA CTG TGG     284
      Thr Lys Leu Ala Val Phe Arg Lys Gln Pro Asp Val Ser Ala Ile Leu Tyr Tyr Lys Asn Leu Glu Glu Leu Trp

1079  CTG GAC TGC GCT GAG AAG CTT ACA CAA AGC GCC TCC TTT GAG CTG AAA ATA ATC GGA TTT GCT AAG CTG CAG GAT     315
      Leu Asp Cys Ala Glu Lys Leu Thr Gln Ser Ala Ser Phe Glu Leu Lys Ile Ile Gly Phe MET Arg Ser Gln Asp
      (gtacg..v..cctcg)

1172  CAG ATA TTA CTG GCC ACA GCG CCC TCC TTT GAG CTG CTT CTG ACA CTC CTT GAT CTC GAA CGG GTT CTC TAC CGC     346
      Gln Ile Leu Leu Ala Thr Gly Ser Thr Gly Pro Gln Leu Leu Asn MET Ser Arg Asn Leu Asn Ser Leu Tyr Gly
                                                                                          (G)

1265  GAC GTG ATG CTG CCC CAG GAG GCG TTC TAC ACA TCC GAA GAG GAG GTG TCG GAA ATC TTC CAA ACG AAG TCG ATA GCC     377
      Asp Val MET Leu Pro Gln Glu Ala Phe Tyr Thr Ser Glu Glu Glu Val Ser Glu Ile Phe Gln Thr Lys Ser Ile Ala
                                                                              (gtacg..v..cctcag)

1358  GAA CTC AAA CTG ACT GGA ACC GGA ATT GGA GTG CCA GAA GCC AAT GGA GTG CGT AAT ACG GAA ATA     408
      Glu Leu Lys Leu Thr Gly Thr Gly Ile Gly Val Pro Glu Ala Asn Gly Val Arg Asn Thr Glu Ile

1451  CAG CTT TTC AAC AAT CTG AGG ATC ATC TCC ATC CAC GGA GTG GAG GAT CTG AGC AAG TTC CTG CGG CGG CAC ACA     439
      Gln Leu Phe Asn Asn Leu Arg Ile Ile Ser Ile His Gly Val Glu Asp Leu Ser Lys Phe Leu Arg Arg His Thr

1544  CTG CTG AAC AAT ATA ATA ATC GAC GAT ATT TCC ATC ATG CAC ATG CTG GAC AGC AAG TTC CAG CAG AAT GTC GTT TTT     470
      Leu Leu Asn Asn Ile Ile Ile Asp Asp Ile Ser Ile MET His MET Leu Asp Ser Lys Phe Gln Gln Asn Val Val Phe
      (gtgag..v..ttcaag)

1637  CCG CCG TAC TAC AAG CAG CTG CTG TCG ATA GAT GAT CAG CAG GAC ACA TAA CAAGACGACACGACCCGACGGCAGCACCATTGCGTAACACTGCACTGC     497
      Pro Ala Leu Tyr Tyr Lys Gln Leu Phe Asp Ser Ile Asp Asp Gln Gln Asp Thr -

1742  CCGCTGCCGCCACGATGTGCTCGCCGGCCGCACCGACCGCACGCGCTGCGGCTCNCGGCCCGCAGTGCGGCACANCAATAATTATTTCGTAAACACTCCACTGC
1866  CCATCACGCATGCNCGACNCTTTATCATGATTTAACCTAACGCTCAACCAAGGATGTCATCCTCCCCAAGGACGCTCATCCTATATACCATATAATAAATATATCACAG
1990  ACGGATGACGCGAAGCGGAGGGAAAATATATTCAAAATATTGGTTAACTCAGTTAAGGACTTTTCGTCAGTTAAGAACCCGCAACCGAAGCCGCATCAAACTGATT
2114  TTCGAGGTTATATCTATTCATAATNCACAGAACACACCACCAGCACACATATAATATATATGTAACTTCCAAACTTCTCAAACTTCTATATCCTCGGCCCGCAGTCAGATCAGATCACTGGTCTTTAGTACTTAAA
2238  ACCAACCGAANTTCTCTACNCGCNCANCGCCGGCCCGTNGCCCCAATAATTCAGTTCCGTTAGTGTTACCCNGANCCCCGGATTCGCGCCTT....

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A

DHR3	51	CRVCGDKSSGVHYGVITCEGCKGFFRRSQSSVV--NYQCPRNKQCVVDRVNRNRCQYCRLQKCLRLGH
EcR	264	CLIVCGDRASGYHYNALTCEGCKGFFRRSVTKSA--VTCHHRGRACEMDMYMRRKCQECRLRKCLAVGH
E75A	245	CRVCGDKASGFHYGVHSCEGCKGFFRRSIQKNI-QTRPCTKNQQCSILRIHRNRCQYCRLRKCIAVGH
knl	5	CRVCGRPLAGFHFGVAFCEACKSFFGRSITKNIS-RISKCKNEGKCIIDKKNRTACKACRLRKCINVGH
hRARα	58	CFVCQDKSSGYHYGVSACEGCKGFFRRSIQKNMVYTCHRDKNCIIMKVTRNRCQYCRLQKCFEVGH
hTRβ	102	CGVCGDRATGYHYRCITCEGCKGFFRRTIQKNLHPTYSCKYDGCCVIDKITRNQCQACRLRRCIDIGH
hVDR	24	CGVCGDRATGFHFNAMTCEGCKGFFRRSMKRKALFTCPFNGDCRITKDNRRHCQACRLRRCVDIGH
COUP-TF		CVVCGDKSSGKHYGQFTCEGCKSFFKRSVRRNLTYTCRANRNCQIDKRQRNRCQYCRYQKCLAMGM
hRXR1	175	CLICGDVASGYHYGVASCEGCKGFFRRSIQKNMVYTCHRDKNCVINKVTRNRCQYCRLQKCIAVGH
hRXR2	103	CLICGDRASGYHYGVSACEGCKGFFRRSIQKNMVYTCHRDKNCVINKVTRNRCQYCRLQKCIAVGH
hER	185	CAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRKSCQACRYRKCYEVGM
hGR	421	CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQH--NYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGM
hMR	603	CLVCGDEASGCHYGVVTCGSCKVFFKRAVEGQH--NYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
hPR	567	CLICGDEASGCHYGVLTCGSCKVFFKRAMEGQH--NYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGM

E1

B

DHR3	255
EcR	431
E75A	380
hRARα	170
hTRβ	238
hVDR	198
COUP-TF	
hRXR1	294
hRXR2	211
hER	315
hGR	531
hMR	737
hPR	686

E2   E3

DHR3	380
EcR	557
E75A	503
hRARα	292
hTRβ	361
hVDR	325
COUP-TF	
hRXR1	410
hRXR2	328
hER	437
hGR	653
hMR	859
hPR	808

Figure — sequence alignment panels A, B (E1, E2, E3 regions) and C (percent identity matrix of DHR3, EcR, E75A, knl, hRARα, hTRβ, hVDR, COUP-TF, hRXR1, hRXR2, hER, hGR, hMR, hPR).

A

DHR3	51	CKVCGDKSSGVHYGVITCEGCKGFFRRSQSSVV--NYQCPRNKQCVVDRVNRNRCQYCRLQKCLKLGH
EcR	264	CLVCGDRASGYHYNALTCEGCKGFFRRSVTKSA--VYCCKFGRACEMDMYMRRKCQKCRLKKCLAVGH
E75A	245	CRVCGDKASGFHYGVHSCEGCKGFFRRSIQQKI-QIRPCTKNQQCSILRINRRRCQYCRLKKCIAVGH
kni	5	CRVCGEPAAGFHFGAFTCEGCKSFFGRSYNNIS-TISECKNRGKCIIDKRNRTTCKACRLRKCYVVGH
hRARα	58	CFYCQDKSSGYHYGVSACEGCKGFFRRSIQKNM--VYTCHRDKNCIINKVTRNRCQYCRLQKCFEVGH
hTRβ	102	CVVCGDKATGYHYRCITCEGCKGFFRRTIQKNLHPSYSCKYEGKCVIDKVTRNQCQKCRFKKCIYVGH
hVDR	24	CGVCGDRATGFHFNAMTCEGCKGFFRRSMKRKA--LFTCPFNGDCRITKDNRRHCQACRLRCVDIGH
cOUP-TF		CVVCGDKSSGKHYGQFTCEGCKSFFKRSVRRML--TYTCRANRNCPIDQHHRNQCQYCRLKKCLKVGH
hERR1	175	CLVCGDVASGYHYGVASCEACKAFFKRTIQGSI--EYSCPASNECEITKRRRKSCQACRFTKCLRVGH
hERR2	103	CLVCGDIASGYHYGVASCEACKAFFKRTIQGSI--EYSCPATMKCEITKRRRKSCQACRFMKCLAVGH
hER	185	CAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHN--DYMCPATNQCTIDKNRRKSCQLCRLRKCYVGH
hGR	421	CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQH--NYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGM
hMR	603	CLVCGDEASGCHYGVVTCGSCKVFFKRAVEGQH--NYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
hPR	567	CLICGDEASGCHYGVLTCGSCKVFFKRAMEGQH--NYLCAGRNDCIVDKIRRKNCPACRIRKCCQAGM

B

E1

DHR3	255	KLRAVHDMFRKQPDVSRILYYKMLGQRLWLDCAKKLTQMIQNIRYAKLLPGTARLSQDDQILLLKKTGSYKLATVRMSRL--LDLS
EcR	431	QDGIKQFSEDLRRLKMSQPDEMRSQTDVSTRHITEITILTVQLIVEFAKGLPAFTKIPQEDQITLLKACSSEVMMLRMARR--YDHS
E75A	380	QRARDCPSYSMPTLLACPLMPAPELQSEQKF--SQRPAHVIRGVID/AGHDPGYQLLIQDDKFTILKAGLFDALLVRLICM--FDS°
hRARα	170	PALCQLGKYTTNSSEQRVSLDIDL--WDKF--SELSYKCIIKIVEFAKQIPGFTTLTIADQITLLKAALCLDILILRICTR--YT.
hTRβ	238	PKFLPDIGQAPIVMAPEGG-KVDLEAFSEF--TKIITPAITRVVDFAKKLPMFCELPCEDQIILLKGCCMEDSLRAAVR--YDP1
hVDR	198	DSSSFSNLDLSEEDSDDPSVTLELSQLSMLPHLADIVSYSIQKVIGFAKMIPGFRDLTSEDQIVLLKSSAIEVIMLRSNES--FTMD
COUP-TF		GYISLLLRAEFYPTSRYGSQCMQPNNDGIENICHLAARLLFSAVEWARMIPFTPDLQITREIVVTISWAKSIPGFSSLSLSDQMSVLQSVWMEVLVLGVAQRSLPLQD
hERR1	294	LVSHLLV-VEPEKLYAMPDPAGPDGHLPAVATLCDLFDREIVVTISWAKSIPGFSSLSLSDQMSVLQSVWMEVLVLGVAQRSLPLQD
hERR2	211	IVSYLLV-AEPDKLYAMPFDDVPEGDIKALTTLCDLADRELVFLISWAKHIPGFSNLTLGDQMSLLQSAWMEILILGIVYRSLPYDD
hER	315	MVSALLD-AEPPILYSEYDPTRPFSELSMMQLLTNLADRELVHMINWARVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHP-
hGR	531	TLVSLLKVIEPEVLYAGYDSSVPDSTWRIDTTAMLGGRQVIAAVKWAKAIPGFRNLHLDDQMTLLQYSWMFLMAFALGWRSYRQSS
hMR	737	SPVMVLENIEPEIVLAGYDSSKPDTAENLLSTLNRLAGKQMIQVVKWAKVLPGFKNLPLEDQITLIQYSWMCLSSFALSWRSYKHTN
hPR	686	PLINLLMSIEPDVIYAGHDNTKPDTSSSLLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVS

E2

```
-LDLSQNAVLYGDVMLPQRAFYTS--DSKERRLVSRIFQTAKSIAEL   DHR3     380   KL------TETELALYQSLVLLMPE-RNGVRGNTEIQRLF
-YDHSSDSIFFANNRSYTRDSYKM-AGADMIRDLLHFCRQMFSMKV    EcR      557   --------DNVEYALLTAIV-IFSD-RPGLEKAQLVEAIQ
-FDSSINSIICLN-GQVMRRDAIQ-MGAMARPLVDSTFNFAERMNSM   E75A     503   NL------TDARIGLFCAIVLITPD-RPGLRNIELIERMY
-YTPKQDTMFTSDGLTLNRTQMEN-AGTGPLTDLVFAFANQLLPLEM   hRAR     292   --------DDARTGILSAICLICGD-RQDLEQPDRVDHLQ
-YDPESETLTLNGRMAVIRGQLKN-GGLGVVSDAIFDLGMSLSSFNL   hTRβ     361   --------DDTEVALLQAVLLMSSD-RPGLACVERIEKYQ
-FTMDDHSWTCGNQDYKYRVSDVTKAGHSLELIRPLIKFQVGLKKLN   hVDR     325   LH------EKHVLLMAICIVSPD-RPGVQDAALIBAIQ
MPLHVAPLLAAAGLHASPHSADRV-VAFHDH-------IRIFQEN     cOUP-TF        VEKLKALHVDSARYSCLKAIVLFTSD-ACGLSDAAHIKSLQ
LPLQDE--LAFABDLVLDERGARA-AGLGEL--------GAALLQL    hERR1    410   VRRLQALRLEREEYVLLKVLALANSD------SVHIR-DE
LPYDDK--LAYAEDYIMDKEHSRL-VGLLEL--------YRAILQL    hERR2    328   VRRYKKLVEKRFVMKLALALANSD------SHYIENLE
MRHP-VKLL-FAPNLLLDRNQGKC-VEGMVE---IFDMLLATSSRFR   hER      437   MMNLQ----GERFVCLKSIILLNSG------VYTFLSST
YRQSSANLLCFAPDLIINE-QRNT-LPCNIDQCKH-----MLYVSSE   hGR      653   LHRLQ----VSYEEYLCMKTLLLLSSVPKDGL-------
YKHTNSQFLTFAPDLVFNE-RKMH-QSMYELCQG------MRQISLQ   hMR      859   FVRLQ----TFKYTDMKVLLLLSTIPKDGL--------
YKHVSGQMLYFAPDLIINE-QRMK-ESSFYISLCLT----MRQIPQE   hPR      808   FVKLQ----VSQRRFLCMKVLLLLNTIPLEGL-------
```

E3

```
DHR3     EIQRLFNLSMNAIRQ------------------------------BLETNHAPLKGDVTVLDTILNNIPNFRDISILEMSLSKYKLQHPN-----VVFPALYKELFS
EcR      LVEAIQSYYIDTLRI------------YILNR----ECGDSMSLVFYAKLLSILTELRTLGNQNAEMCFSLKLKNVRK-----LPKFLEEIWD
E75A     LIRKMYSRLKGCLQ------------YIVAQ-----NRPDQPKFLAKLLETMPDIARTLSTLHTEKL-----VVFRTEHKELLR
hRAR     RVDMLQEPLLEALKV------------Y-VRK-----RRPSRPHMFPKMLMKITDLRSISAKGAERVITLKMEIPGSM----PPLIQEMLEN
hTRβ     RIERKYQDSFLLAFEH------------YINYR-----KHHVTHFWPKLLMKVTDLRMIGACHASRFLHMKVECPTELL----PPLFLEVFED
hVDR     LIKAIQDRLSNTLQT------------YIRCREPPGSHLLYAKMIQKLA----DLRSLNDDHSKQYRCLSFQ-PEC-SMKLTPLVLEVFGN
cOUP-TF  HIHSLQEKSQCALEE------------YVRSQ-YPNQPSRFGKLLLRLPSLRTVSSSVIEQLFFVRLVGKTPIE-TLIRDMLLSGSS
hERR1    HIE-DEPRLMSSCH-KLLHEALLEYE------------AGRAGPGGGAERRRAGRLLTLPLLRQTAGKVLAHFYGVKLEGKVPMH-KLFLEMLEAMMD
hERR2    YIENLBAVQKLQ------------DLLHEALQDYE------------LSQRHEEBPRRAGKLLLTLPLLRQTAAKAVQHFYSVKLQGKVPMH-KLFLEMLEAKV
hER      TFLSSTLKSLE------------EKDHIHRVLDKITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLY-DLLLEMLDAHRL
hGR      ------KSQELFDEIRMFYIKELGK------------AIVKREGNSSQNWQRFYQ-LTKLLDSMHEVVENLLN----YCQQTFLD-KTMSIEFPEMLAEIIT
hMR      ------KSQAAFEMRTHYIKELRK------------HVTKCPNNSGQSWQRFYQ-LTKLLDSMHDLVSDLLE----FCFYTFRESHALKVEFPAMLVEIIS
hPR      ------RSQTQFEEMRSSYIRELIK------------AIGLRQKGVVSSSQRFYQ-LTKLLDNLHDLVKQ-----LHLYCLNTFIQSRALSVEFPEMMSEVIA
```

C

	DHR3	EcR	R75A	kni	hRARα	hTRβ	hVDR	cOUP	hERR1	hERR2	hER	hGR	hMR	hPR
hPR	15	13	15		16	15	13	20	25	25	27	54	56	
hMR	16	14	12		15	18	15	19	23	21	25	57		91
hGR	13	14	14		14	14	13	21	25	25	27		94	91
hER	15	17	13		17	19	15	24	32	33		58	50	56
hERR2	14	15	15		18	17	19	30	63		72	57	59	54
hERR1	13	16	15		18	18	20	29		91	69	57	59	54
cOUP	14	20	16		18	16	17		51	54	55	48	52	45
hVDR	17	25	22		22	22		50	45	45	47	41	44	42
hTRβ	18	29	20		33		52	59	56	57	53	44	45	42
hRARα	18	24	23			62	47	61	53	54	59	45	48	44
kni					45	52	53	50	43	43	52	45	47	45
R75A	24	23		48	62	58	55	58	54	54	58	44	47	44
EcR	19		55	48	55	59	58	58	53	51	52	48	50	47
DHR3		55	64	47	65	56	50	62	48	49	53	47	52	48

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WHAT IS CLAIMED IS:

1.   An isolated recombinant nucleic acid which, upon expression, is capable of coding for other than a native vertebrate steroid receptor or fragment thereof, said nucleic acid comprising a segment having a sequence substantially homologous to a coding region of domains A, B, D, E or F from an insect steroid receptor superfamily member gene having substantial homology to a steroid binding domain.

2.   An isolated recombinant nucleic acid of Claim 1, wherein said insect steroid receptor superfamily member is EcR, DHR3, E75A or E75B.

3.   An isolated recombinant nucleic acid of Claim 1, wherein said nucleic acid encodes a polypeptide capable of binding to a ligand for an insect steroid receptor superfamily member.

4.   An isolated recombinant nucleic acid of Claim 1, wherein said nucleic acid is capable of hybridizing to an insect steroid receptor superfamily member gene segment under selective hybridization conditions.

5.   An isolated recombinant nucleic acid of Claim 4, wherein said selective hybridization conditions are stringent hybridization conditions.

6.   A cell transformed with an isolated recombinant nucleic acid of Claim 1.

7.   An isolated recombinant nucleic acid having a sequence exhibiting identify over 20 nucleotides of a coding segment of an insect steroid receptor superfamily member having steroid binding domain homology.

8.   An isolated recombinant nucleic acid of Claim 7, wherein said nucleic acid encodes a polypeptide which binds to a control element responsive to a ligand of an insect steroid receptor superfamily.

9.   A cell transformed with an isolated recombinant nucleic acid of Claim 7.

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10.   An isolated recombinant nucleic acid comprising a DNA sequence capable of binding to an insect steroid receptor superfamily member other than 20-OH ecdysone receptor.

5   11.   An isolated recombinant nucleic acid of Claim 10, wherein said insect steroid receptor superfamily member is DHR3, E75A or E75B.

12.   An isolated recombinant nucleic acid of Claim 10, wherein said DNA sequence promotes
10   transcription of an operably linked sequence in response to binding by said insect steroid receptor superfamily member.

13.   An isolated recombinant nucleic acid of Claim 10, wherein said DNA sequence is operably linked to
15   a DNA sequence encoding a polypeptide.

14.   An isolated recombinant nucleic acid of Claim 10, wherein said isolated recombinant nucleic acid is an expression vector.

15.   A cell transformed with an isolated recombinant
20   nucleic acid of Claim 10.

16.   A cell of Claim 15, wherein said cell also comprises said insect steroid receptor superfamily member.

17.   A recombinant nucleic acid comprising:
25   a control element responsive to a ligand of an
             insect steroid receptor superfamily member
             ligand responsive control element;
         a non-heat shock promoter sequence; and
         a sequence comprising a reporter gene.

30   18.   A recombinant nucleic acid of Claim 17, wherein said non-heat shock promoter sequence is an alcohol dehydrogenase promoter.

19.   A cell transformed with a recombinant nucleic acid of Claim 17.

35   20.   A cell of Claim 19, wherein said cell is a mammalian cell.

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21.   A recombinant nucleic acid of Claim 17, comprising additional copies of control elements responsive to a ligand of an insect steroid receptor superfamily member.

22.   A method for monitoring expression of a reporter gene comprising the steps of: expressing a recombinant nucleic acid of Claim 17, wherein no radioactive reagent is used in said monitoring of said reporter gene.

23.   A polypeptide comprising an insect steroid receptor superfamily member or fragment thereof, wherein said polypeptide is substantially free of naturally-associated insect cell components and exhibits a biological activity characteristic of an insect steroid receptor superfamily member with a steroid binding domain.

24.   A polypeptide of Claim 23, wherein said insect steroid receptor superfamily member is selected from the group consisting of EcR, DHR3, E75A and E75B.

25.   A polypeptide of Claim 23, wherein said insect steroid receptor superfamily member or fragment thereof also comprises a DNA binding domain.

26.   A polypeptide of Claim 23, wherein said insect is Drosophila melanogaster.

27.   A polypeptide of Claim 23, which is capable of binding to a hormone analogue selected from the group consisting of an insect hormone and an insect hormone agonist.

28.   A polypeptide of Claim 27, wherein said insect hormone is an ecdysteroid.

29.   A polypeptide of Claim 27, wherein said insect hormone is 20-OH ecdysone.

30.   A polypeptide of Claim 23, which is capable of binding to a DNA control element responsive to an insect hormone.

31.   A polypeptide of Claim 30, wherein said polypeptide comprises a zinc-finger domain.

32.   A polypeptide of Claim 30, wherein said insect hormone responsive DNA control element is operably linked to a transcription unit which is responsive to said binding.

33.   A polypeptide of Claim 32, wherein said insect hormone responsive DNA control element is upstream from said transcription unit.

34.   A polypeptide of Claim 23 fused to a second polypeptide.

35.   A polypeptide of Claim 34, wherein said second polypeptide is a heterologous polypeptide.

36.   A polypeptide of Claim 35, wherein said heterologous polypeptide comprises a second steroid receptor superfamily member.

37.   A polypeptide of Claim 23, wherein said fragment has a sequence substantially homologous to a consensus E1 region sequence.

38.   A polypeptide of Claim 23, wherein said fragment has a sequence substantially homologous to a consensus E2 region sequence.

39.   A polypeptide of Claim 23, wherein said fragment has a sequence substantially homologous to a consensus E3 region sequence.

40.   A polypeptide of Claim 23, wherein said fragment has a sequence comprising:

    a segment at least about 25% homologous to a
        consensus E1 region sequence;

    a segment at least about 30% homologous to a
        consensus E2 region sequence; and

    a segment at least about 30% homologous to a
        consensus E3 region sequence.

41.   A composition of matter comprising a polypeptide of Claim 23.

42.   A cell comprising a polypeptide of Claim 23.

43.   A cell of Claim 42, wherein said cell is a human cell.

44.   An antibody or binding fragment thereof
exhibiting binding specificity to a polypeptide of
Claim 23, wherein said binding specificity is directed to
an epitope characteristic of an insect steroid receptor
superfamily member.

45.   A method for selecting DNA sequences capable of
being specifically bound by an insect steroid receptor
superfamily member, said method comprising the steps of:
        screening DNA sequences for binding to a polypeptide
               of Claim 23; and
        selecting said DNA sequences exhibiting said
               binding.

46.   A method of Claim 45, wherein said DNA sequence
is operably linked to a gene selected from the group
consisting of EcR, DHR3, E74 and E75 genes.

47.   A method for selecting ligands specific for
binding to a hormone binding domain of an insect steroid
receptor superfamily member, said method comprising the
steps of:
        screening compounds for binding to one or more
               superfamily members; and
        selecting compounds exhibiting specific binding to
               the members.

48.   A method of Claim 47, wherein said ligand is an
ecdysteroid.

49.   A method of Claim 47, wherein said ligand is a
20-OH ecdysone antagonist.

50.   A method for modulating insect physiology or
development comprising the steps of:
        screening compounds for binding to an insect steroid
               receptor superfamily member;
        selecting said compounds exhibiting said binding;
               and
        administering to an insect said ligand.

51.   A method of Claim 50, wherein said modulating
is lethal to said insect.

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52.   A fusion polypeptide comprising a hormone
binding domain of an insect steroid receptor superfamily
member and a second polypeptide.

53.   A fusion polypeptide of Claim 52, wherein said
5   second polypeptide comprises a DNA binding domain from a
second steroid receptor superfamily receptor member.

54.   A nucleic acid encoding a fusion polypeptide of
Claim 52.

55.   A method for selecting ligands specific for
10   binding to a ligand binding domain of an insect steroid
receptor superfamily member, said method comprising the
steps of:

    combining:

        (i)   a fusion polypeptide of Claim 52, wherein
15   said fusion polypeptide comprises
                said ligand binding domain
                functionally linked to a DNA binding
                domain of a second steroid receptor
                superfamily member; and

20   (ii) a second nucleic acid sequence encoding a
                second polypeptide, wherein
                expression of said second nucleic
                acid sequence is responsive to
                binding by said DNA binding domain;
25   and

    screening compounds for an activity of inducing
        expression of said second polypeptide; and
    selecting said compounds.

56.   A method of Claim 55, wherein said combining
30   occurs within a cell.

57.   A method of Claim 56, wherein said combining
step results from expression upon transformation of said
cell with a nucleic acid encoding said fusion
polypeptide.

35   58.   A method for modulating insect physiology
comprising the step of:

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administering to an insect a ligand selected by a
method of Claim 55.

59.   A method of Claim 55, wherein said binding
domain is selected from binding domains of insect steroid
receptor superfamily members selected from the group
consisting of EcR, DHR3, E75A and E75B.

60.   A method of Claim 58, wherein said ligand is
lethal to said insect.

61.   An isolated receptor control element non-
responsive to 20-OH ecdysone comprising a DNA segment
capable of binding to an insect steroid receptor
superfamily member and capable of controlling expression
of an operably linked gene.

62.   An isolated receptor control element of
Claim 61, wherein said operably linked gene is within
about 50 kb of said control element.

63.   An isolated receptor control element of
Claim 61, wherein said insect steroid receptor
superfamily member is DHR3, E75A or E75B.

64.   A method for producing a polypeptide comprising
the steps of:

    selecting a cell which is substantially insensitive
       to exposure to an insect steroid receptor
       superfamily ligand;

    introducing into said cell:

       (i)   a receptor for said ligand; and

       (ii)   a nucleic acid sequence encoding said
           polypeptide, said nucleic acid
           sequence operably linked to a
           control element responsive to
           presence of said selected
           ligand, wherein a transformed
           cell is produced; and

    exposing said transformed cell to said ligand.

65.   A method of Claim 64, wherein said cell is a
mammalian cell.

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66.   A method of Claim 64, further comprising the step of introducing said cell into an intact organism.

67.   A method of Claim 64, wherein said cell is a plant cell.

5   68.   A method of Claim 64, wherein said ligand is 20-OH ecdysone.
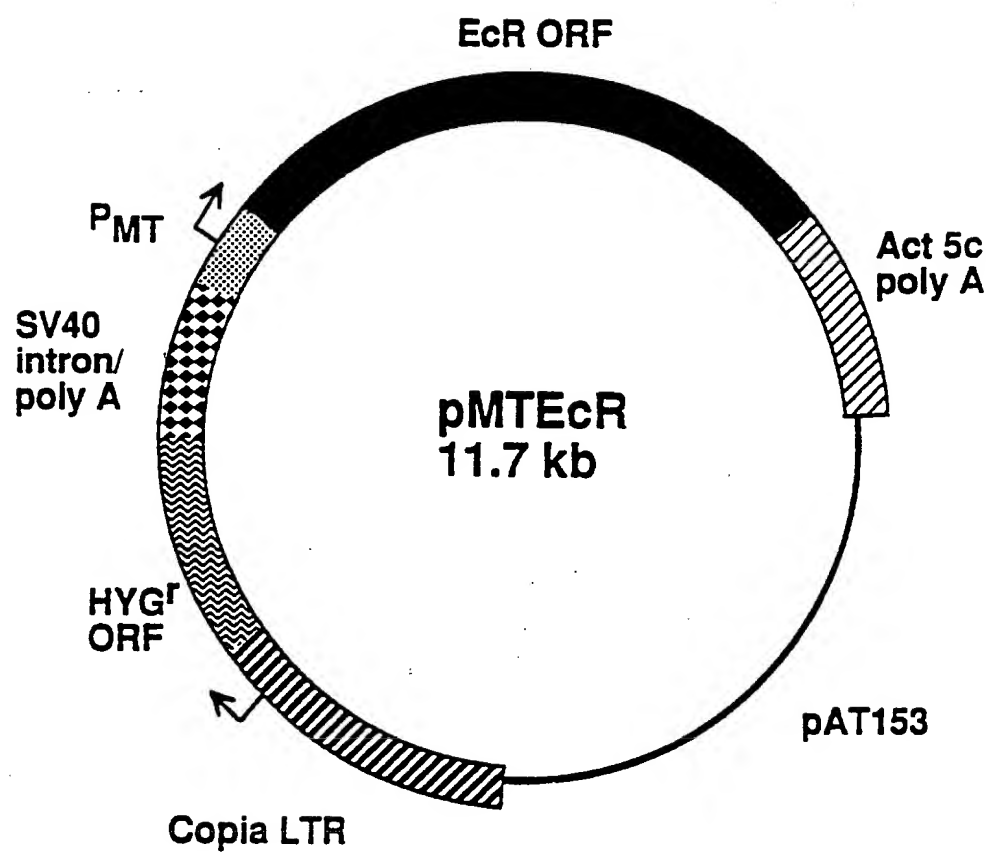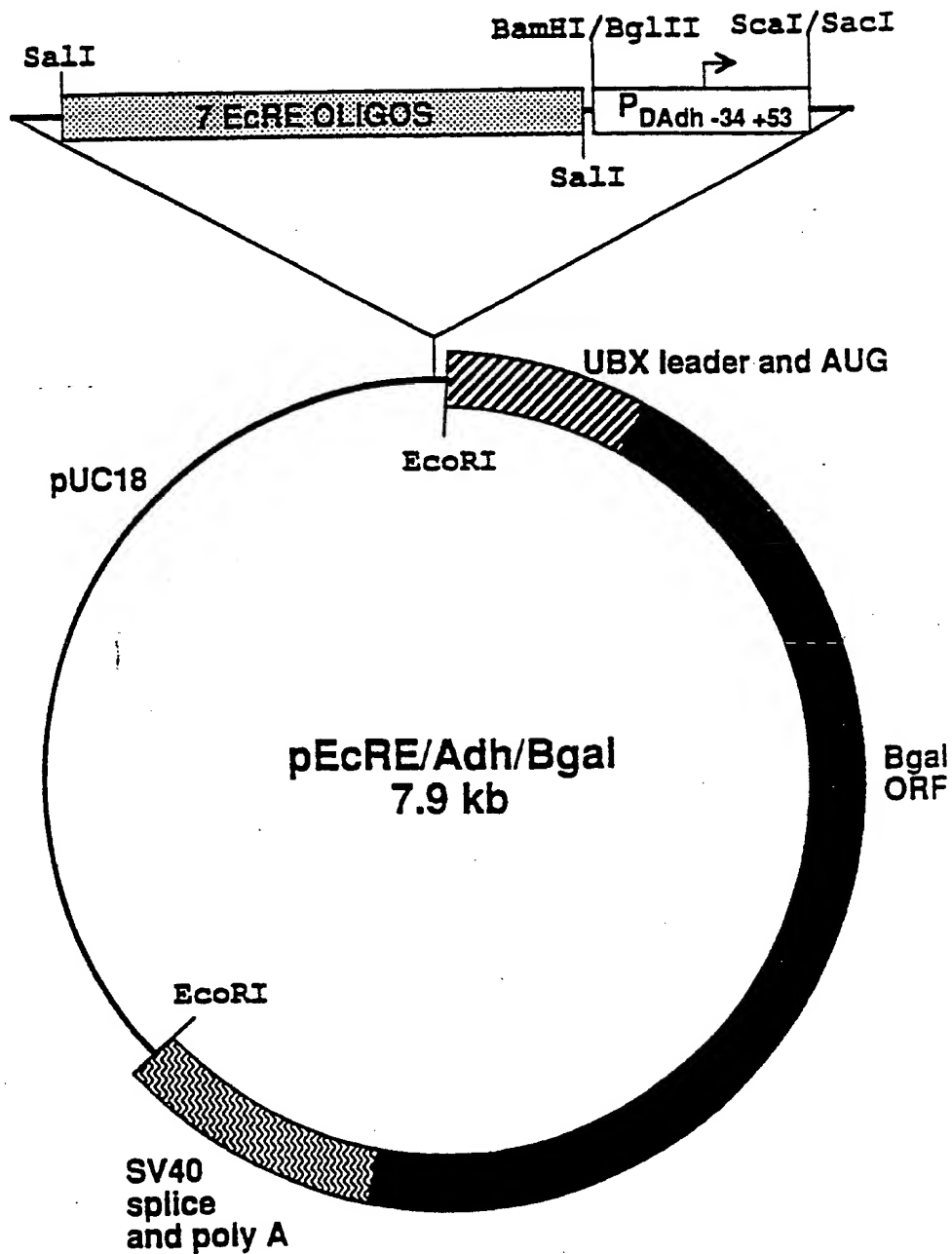
b:\5490A-76.PCT

FIG._1.

FIG._2.

SUBSTITUTE SHEET

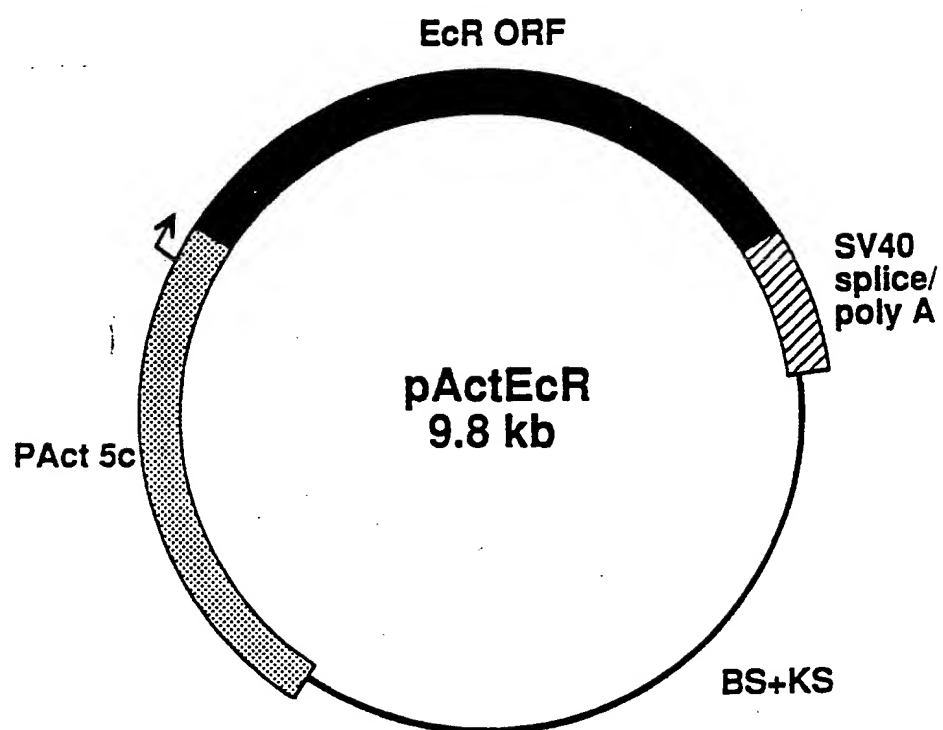*FIG.__3.*

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION SUBJECT MATTER (if several classification symbols apply, indicate all)
According to International Patent Classification (IPC) or to both National Classification and IPC
U.S. CL.: 435/69.1; 530/387; 536/27
IPC(5): C12P 21/06; A61K 35/14; C07H 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System	Classification Symbols
U.S. CL.	435/69.1; 530/38.7; 536/27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

Chemical Abstracts, Biological Abstracts

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	European Journal of Biochemistry. volume 189. issued 1990. M. Strangmann-Diekmann et al. "Affinity Labelling of Partially Purified Ecdysteroid Receptor with Bromoacetylated 20-OH-ecdysone Derivative". pages 137-143. see pages 138 and 141-143.	23-43
X	Molecular and Cellular Endocrinology. volume 57. issued 1988. M. Lehmann et al. Ecdysteroid Receptors of the blowfly. Calliphora vicina: Partial Purification and Characterization of Ecdysteroid Binding". pages 239-249. see pages 242. 244 and 246-248.	23-43
A	T. Maniatis et al. "Molecular Cloning. A Laboratory Manual" published 1989. Cold Spring Harbor Laboratory Press (N.Y.). see pages 8.60-8.63.	1-5 7.8

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31 May 1991	27 JUN 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Deborah Crouch

Form PCT/ISA/210 (second sheet) (Rev 11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET	

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers   : because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers   , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

    See attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

    Claims 1-5, 7-8, 23-43   telephone practice

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)

LACK OF UNITY OF INVENTION

PCT/US91/01189

I.   Claims 1-5 and 7-8; insect steroid receptor DNA; classified
      in 536/27

II.   Claims 6 and 9; cells transformed with insect steroid
      receptor DNA; classified in 435/69.1

III. Claims 10-14; DNA sequence binding to insect steroid
      receptor DNA; classified in 536/27

IV.   Claims 15 and 16; cells transformed with DNA sequence
      binding to insect steroid receptor; classified in 435/69.1

V.   Claims 17-21; DNA construct; classified in 536/27

VI.   Claim 22; method for monitoring expression; classified in
      435/6

VII. Claims 23-43; a polypeptide; classified in 530/350

VIII. Claim 44; antibody; classified in 350/387

IX.   Claims 45-49; method to select DNA sequence which binds
      insect steroid receptor; classified in 435/6

X.   Claims 50, 51, 58 and 60; method to modulate insect
      physiology; classified in 424/405

XI.   Claims 52 and 53; fusion polypeptide; classified in 530/350

XII. Claim 54; nucleic acid; classified in 536/27

XIII. Claim 55-57 and 59; method for selecting ligands; classified
      in 436/501

XIV. Claims 61-63; receptor control elements; classified in
      536/27

XV.   Claims 64-68; method to produce a polypeptide; classified in
      435/69.1

     Groups I, II, III, IV, V, VII, VIII, XI, XII and XIV are distinct
products. Groups VI, IX, X, XIII and XV are distinct methods. The
claims of these groups are drawn to distinct inventions, which
are not linked to form a single inventive concept. PCT rules 13.1
and 13.2 does not allow an application to contain more than one
inventive concept. All other products, methods of making and
methods of using are additional invnetions.